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Award Number: DAMD17-96-1-6231

TITLE: C-7 Progesterone Analogues and MDR1 in Breast Cancer

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REPORT DATE: September 2001

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 2001	Final (1 Sep 96 - 31 Aug 01)

4. TITLE AND SUBTITLE
C-7 Progesterone Analogues and MDR1 in Breast Cancer

5. FUNDING NUMBERS
DAMD17-96-1-6231

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

We have generated novel analogs of progesterone (PgA) as potent inhibitors of the MDR1 multidrug resistant phenotype in breast cancer. The objectives of this Research Project include the optimization of the PgA's MDR1-reversing activity through the generation of additional compounds, using an analog design approach, the definition of these compounds' *in vivo* efficacy, and of their mechanism of action. In the course of the fourth year of the Project, we have: 1. tested the *in vivo* MDR-reversing activity of PgA4, the most potent among our initial progesterone analogs; 2. designed and synthesized additional progesterone analogs and evaluated their structure-activity relationships; 3. related the *in vitro* activity of some of the analogs to their *in vitro* toxicity.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast Cancer	151
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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TABLE OF CONTENTS

INTRODUCTION

Resistance to available chemotherapy is central to our failure to cure many advanced breast cancers. The mechanisms responsible for drug resistance in breast cancer are likely to be multiple. However, expression of the protein product of the MDR1 gene P-glycoprotein (Pgp), appears to correlate with a more than 3-fold increase in the relative risk of breast cancers to fail to respond to systemic chemotherapy [1]. Pgp is a membrane glycoprotein whose expression *in vitro* confers a multidrug resistant phenotype, apparently by an active efflux mechanism from the cell membrane bilayer [2]. Pgp substrates include several critical anticancer agents including anthracyclines, taxanes, vinca alkaloids and epipodophyllotoxins [3]. Inhibition of the resistance to anticancer substrates conferred by Pgp has been demonstrated for several compounds [4], several of which may act by a competitive mechanism [5-12]. Relatively few of these agents have reached clinical trial [13]. These include "first generation" MDR1-reversing drugs originally designed for different purposes (e.g., verapamil and cyclosporin A), whose efficacy is often limited by toxicity related to their original pharmacological purpose [14-17]. "Second generation" MDR1 inhibitors include drugs structurally related to first generation drugs, but selected to be less toxic. These include dexverapamil, dexniguldipine and S9788, drugs whose use is anyway limited by cardiovascular toxicity [18-21]. Finally, "third generation" drugs are supposedly the result of a targeted drug discovery approach. Perhaps, the most advanced in clinical trial is the cyclosporin analog SDZ PSC 833 (Valspodar). Evidence of activity of this drug has been obtained in refractory or relapsed multiple myeloma and acute myelogenous leukemia [22, 23]. However, SDZ PS 833 administration can cause hyperbilirubinemia (frequently) and, sporadically, severe ataxia [23, 24]. This drug has since been withdrawn from clinical trials. The toxicities observed so far highlight the need for more rationally designed agents with improved therapeutic index.

We have used a rational analog-based approach to the design of new and more effective MDR1-reversing agents. We have selected a natural and relatively non-toxic steroid, progesterone as our lead compound. Progesterone is the most potent inhibitor of the MDR1 phenotype among major physiological steroids [25]. We introduced modifications based on the available knowledge about the structural determinants of both MDR1 and of the steroid hormonal activity [26], and our own work presented in the original proposal. We designed and synthesized progesterone analogs where a bulky side chain, including one or two aromatic rings and a urea group, is substituted on the C7 position of the steroidal nucleus. In preliminary evaluations, these novel analogs of progesterone (PgA) showed not only an increased MDR1 reversing activity (up to 35 fold higher than the parental compound, in terms of both chemosensitization and increased cell accumulation of vinblastine), but also decreased progesterone agonist and glucocorticoid agonist/antagonist activities. The most favorable ratio of MDR1-reversing to hormonal activity was observed with PgA4, an analog where the C7 side-chain includes two aromatic rings (rings E and F) connected by a urea-containing bridge (data presented in the original Proposal).

This research project was dedicated to the optimization of the MDR1 reversing activity of C-7 progesterone analogs, to the investigation of their mechanism of action and to the evaluation of the *in vivo* efficacy of one of the analogs (PgA4).

BODY OF REPORT

METHODS

Cell lines.
For our *in vitro* and *in vivo* experiments we used cells transduced with a retroviral vector directing the constitutive expression of the Pgp gene (MDA435/LCC6^{MDR1}) and their parental, Pgp-negative MDA435/LCC6 human breast cancer cells. Both MDA435/LCC6 and MDA435/LCC6^{MDR1} cells are estrogen and progesterone receptor negative, grow as monolayer cultures *in vitro*, and as rapidly proliferating solid tumors and malignant ascites *in vivo* in nude mice [27]. The cells were routinely grown *in vitro* in Improved Minimal Essential Media (Biofluids) containing 5% fetal bovine serum in a 5% CO_2: 95% air atmosphere. For some of our in vivo experiments we also used Pgp-positive P388/ADR murine leukemia cell line. This cell line was obtained by selection of Pgp-negative P388 cells with doxorubicin [28]. These cells were cultured in RPMI 1640 growth media containing 10% FCS.

Evaluation of *in vitro* MDR1-reversing activity: Doxorubicin Accumulation Assay
The MDR1-reversing activity of all new agents was evaluated in terms of their effect on doxorubicin accumulation in MDA435/LCC6^{MDR1} human breast cancer cells. Pgp-negative MDA435/LCC6 were used as a negative control and to evaluate non-specific effects. MDA435/LCC6 and MDA435/LCC6^{MDR1} cells were plated at 2.5 x 10^5 cells/well in the wells of 24-well plates, and incubated for 24 hrs at 37 °C in a humidified, 95% air/5% CO_2 atmosphere. 24 hours after plating, cells were treated by exchanging spent media with the media containing the test compounds at 4 different concentrations + doxorubicin 4 μM (0.5 ml/well). All treatments were carried out in triplicate. Cell cultures were then reincubated at 37 °C for 3 hours. Treatments were stopped by carefully washing wells once with 0.5 ml/well ice-cold NaCl (0.15 M). Cells from reference wells in each plate were counted. Doxorubicin was extracted from the cell monolayer in the remaining wells by first adding 0.75 ml dH_2O, and then 0.75 ml 40% trichloroacetic acid per well. Plates were incubated overnight at 4 °C in the dark. For spectrofluorimetry, 1.2 ml of the extract from each well were transferred into 13 x 100 mm borosilicate glass tubes placed in the 10 x 10 rack of a Hitachi A3000 Autosampler. The autosampler was connected to a Hitachi F4500 Spectrofluorimeter. Fluorescence of each sample was read at 500 nm excitation and 580 nm emission wavelengths. The doxorubicin concentration in each sample was calculated by interpolation on a doxorubicin standard curve and normalized by extract volume and number of cells per well.

Analysis of data from accumulation studies. Results were plotted both in terms of the estimated drug concentration per 10^6 cells and as the percentage of drug accumulation differential (difference between accumulation in untreated MDA435/LCC6 and MDA435/LCC6^{MDR1} cells) reversal *vs.* test compound concentration. MDR1-reversing potency was calculated in terms of the chemosensitizer concentration that induces a 50% reduction in the drug accumulation differential (EC_{50}) in the Pgp-positive cells, by interpolation on the dose-response curve. A "MDR1-specific" EC_{50} value was

obtained by interpolation on the dose-response curves corrected by subtraction of the accumulation effect in the Pgp-negative cells.

Evaluation of *in vitro* toxicity.

MDA435/LCC6 and MDA435/LCC6^{MDR1} cells were plated in 96-well plates and, 24 hours later, they were exposed to growth media containing different concentrations of the test agents (progesterone, C7 progesterone analogs, cyclosporin A or verapamil) for 5 days. Cell cultures were then fixed and stained by incubation in a 0.5% (w/v) crystal violet solution in 25% methanol (v/v). After plates had dried, the dye was extracted in 0.1 M sodium citrate in 25% methanol (v/v) and absorbance was read at 540 nm using a microplate spectrophotometer. Absorbance directly correlates with cell number in this assay. Cell survival curves were obtained by plotting absorbance values (as % of untreated controls) against drug concentration. The toxicity of each drug was summarized in terms of IC_{50}, the concentration decreasing cell density by 50% at the end of the treatment period. For those drugs that produced a detectable IC_{50}, the ratio of IC_{50} values in MDA435/LCC6^{MDR1} and MDA435/LCC6 cells provide an estimate of the relative resistance of Pgp-positive cells. Ratios of > 1 are suggestive of a possible transport of the test drugs by Pgp.

Evaluation of UV profile and Specific Absorption Coefficient (E_1^1)

PgA4 was dissolved in methanol at 1 mg/ml. An aliquot from this stock was diluted to 10 µg/ml in methanol. The UV profile of the diluted solution was evaluated using a Beckman DU-460 spectrophotometer by scanning its absorbance between 190 and 390 nm wavelength. Based on PgA4 UV profile, we chose 245 nm as our reference wavelength for future evaluations, as this corresponds to a plateau in the UV profile. The specific absorption coefficient E_1^1, defined as the absorption at path length 1 cm and compound concentration 10 mg/ml, was calculated by multiplying the recorded absorption at 245 nm wavelength by 1000.

Evaluation of PgA4 solubility

A saturating amount of PgA4 was suspended in the test vehicle and was kept shaking overnight, at room temperature. The drug suspension was then spun in a microfuge at 13,200 rpm for 10 min and the supernatant filtered through Micropure separators (Amicon). After a 1:200 dilution in the same vehicle, the filtrates were evaluated for UV absorption at 245 nm. Drug concentration in the undiluted filtrates, assumed to represent its solubility in the specific vehicle, was calculated as:

$$Cs = A \times df \times 10 / E_1^1$$

where Cs represents drug solubility, A the absorption at 245 nm wavelength, df the factor by which filtrates were diluted (=200), and E_1^1 is the specific absorption coefficient and is =376 (as described above).

Evaluation of *in vivo* toxicity.

NCr *nu/nu* female athymic nude mice (two per treatment group) were treated with either PgA4, prepared at 1.5 mg/ml in 20% 2-hydroxypropyl- -cyclodextrin, 15 mg/Kg, or the same amount of vehicle *sc*, twice a day for 3 days. Mice were observed for immediate or delayed signs of toxicity (mortality, altered behavior, decrease in body weight gain).

Evaluation of the effect on anticancer drug on *in vivo* toxicity

Effect of PgA4 on doxorubicin toxicity. We administered PgA4 as described above for the PgA4 toxicity studies, *i.e.*, *s.c* , every 12 hours for 6 times. The dose of PgA4 per inoculation was 28 mg/Kg (*i.e.*, higher than average, in this instance). Doxorubicin (8 mg/Kg) was administered *i.v.* at the time of PgA4's 5th inoculation.

Effect of PgA4 on taxol toxicity. We evaluated the effect of PgA4 on the *in vivo* toxicity of taxol. For this experiment, taxol was administered *i.v.* at 8 mg/Kg weekly for four weeks. PgA4 was administered *s.c.* at a dose of 15 mg/Kg, 6 times weekly: at the time of taxol treatment, and 48, 36, 24, 12 hours before, and 12 hours after taxol treatment.

Evaluation of *in vivo* MDR1 reversing activity.

In most of our experiments we used NCr *nu/nu* female athymic nude mice inoculated either *i.p* (doxorubin accumulation assays) or *s.c.* with MDA435/LCC6 (control) or MDA435/LCC6^{MDR1} cells. However, for survival experiments we used DBA/2 mice inoculated *i.p* with 1x10^6 P388 (Pgp-negative control) or P388/ADR (Pgp-positive) cells. Different endpoints and different schedules of drug administration were used to evaluate MDR1 reversing activity.

Doxorubicin accumulationt endpoint. In these experiments, we used NCr *nu/nu* female athymic nude mice inoculated *i.p* with either 1x10^6 MDA435/LCC6 (control) or MDA435/LCC6^{MDR1} cells. Mice were treated with PgA4 as soon as the ascites started to develop. PgA4, prepared at the maximal achievable concentration in a 20% 2-hydroxypropyl- -cyclodextrin solution, was administered *s.c.*, 10 µl/g body weight, in the interscapular region twice a day for 3 days. Control mice were treated with vehicle only. Doxorubicin, 12 mg/Kg, was administered as a single *iv* inoculation immediately following the 5th inoculation of PgA4. Control mice received an equivalent amount of saline solution. 24 hours after doxorubicin inoculation, the mice were sacrificed. The ascitic fluid was collected and spun in a microfuge at 4000 rpm for 4 min, the relative pellet extracted with 20% trichloroacetic acid and the extract evaluated for doxorubicin content fluorimetrically, as described above for *in vitro* evaluations.

Tumor growth inhibition endpoint. Tumors were obtained by inoculating *s.c.* into the left flank of female NCr *nu/nu* mice 1 x 10^6 MDA435/LCC6^{MDR1} cells from a donor mouse. The same mice were inoculated in the right flank with an equal number of Pgp-negative parental MDA435/LCC6 cells. Treatments were applied starting on the day following cell inoculation. Treatment administration was the same as described above for the evaluation of PgA4 effect on taxol *in vivo* toxicity. Tumor sizes were measured 2-3 times per week. Any increase in the inhibition of tumor growth specific or preferential to MDA435/LCC6^{MDR1} tumors is assumed to represent a cancer cell MDR1-specific effect, while any increase in the inhibition of MDA435/LCC6 tumor growth more likely represents the result of PgA4 interaction with the anticancer drug pharmacokinetics.

Survival endpoint: local administration of PgA4. DBA/2 mice, inoculated *i.p.* with 10^6 Pgp-expressing P388/ADR cells on day 0, were treated by the same route (*i.p.*) with PgA4 1.4 mg/Kg (10 ul/g of body weight of a solution 0.14 mg/ml in 20% 2-hydroxypropyl- -cyclodextrin; in 5 mice) or the equivalent amount of vehicle (5 mice), and doxorubicin HCl 2.5. mg/Kg, on days 1, 5 and 9.

Survival endpoint: systemic administration of PgA4. On day 0, DBA/2 mice, were inoculated *i.p.* with either 10^6 Pgp-expressing P388/ADR cells or Pgp-negative parental P388 cells. 10 mice per cell line were assigned to each of four treatment groups: *i.* Vehicle only (control); *ii.* PgA4 only; *iii.* doxorubicin only; *iv.* PgA4 + taxol. Pga4 was administered *s.c.* 1.4 mg/Kg (or the equivalent amount of vehicle), and doxorubicin was administed *i.p.* at 2.5 mg/Kg (or the equivalent amount of vehicle). Both PgA4 and Doxorubicin were administered on days 1, 5 and 9.

Preparation of immobilized Pgp stationary phase and evaluation of substrate binding by affinity chromatography.

The methodology used to immobilize Pgp in the stationary phase of a chromatographic column, and to evaluate Pgp substrate binding by affinity chromatography, is described in detail elsewhere [29,30]. Briefly, for the preparation of Pgp stationary phase, MDA435/LCC6^{MDR1} cells (from 20 to 200 x 10^6) were harvested in 30 ml of PBS saline and homogenized for 20 sec with a Brinkmann Polytron homogenizer. The homogenates were centrifuged at 35,000 x g for 10 min and the pellets were suspended in 4 ml solubilization solution (50 mM Tris-HCl, pH 7.4, 250 mM NaCl, 0.5% CHAPS, 2 mM DTT, 5% glycerol) and stirred for 1 hr at 0 °C.

200 mg of dried IAM particles were suspended in 4 ml of the receptor-detergent solution and stirred for 1 hour at 4 °C. The mixture was dialyzed against dialysis buffer (150 mM NaCl, 10 mM Tris-HCl buffer, pH 7.4, 1 mM EDTA) for 72 hours at 4 °C. The obtained Pgp-IAM particles were washed with the buffer by centrifugation and packed in a glass column (id 0.5 cm).

We used frontal chromatography to calculate the dissociation constants, K_d, for the marker and displacer ligands. The experimental approach involves the variation of the concentrations of competitive unlabeled ligands (e.g., vinblastine, verapamil, doxorubicin or cyclosporin A) with a fixed concentration of tracer ligand (e.g., [3H]verapamil, [3H]vinblastine or [3H]cyclosporin A). The association constants of T, K_T, and the test drug, K_{drug}, as well as the number of the active and available binding sites of immobilized receptors, P, can be calculated from the retention volumes of the tracer ligands using the following equations, Eqn 1 and Eqn 2.

$$(V_{max} - V)^{-1} = (1+[T]\,K_T)(V_{min}\,[P]\,K_T)^{-1} + (1+[T]\,K_T)^2\,(V_{min}\,[P]\,K_T\,K_{drug})^{-1}\,[drug]^{-1} \qquad \text{(Eqn 1)}$$

$$(V - V_{min})^{-1} = (V_{min}\,[P]\,K_T)^{-1} + (V_{min}\,[P])^{-1}\,[T] \qquad \text{(Eqn 2)}$$

In the above equations, V is the retention volume of T; V_{max}, the retention volume of T at low concentration and in the absence of drugs; V_{min}, the retention volume of T when the specific interaction is completely suppressed. The value of V_{min} is determined by running T in a series of concentration of drugs and plotting $1/(V_{max} - V)$ *versus* 1/[drug] extrapolating to infinite [drug]. From the above plot and a plot of $1/(V - V_{min})$ *vs.* [T], dissociation constant values, K_d, for T and the drugs are obtained.

CHEMISTRY

General Considerations

All reactions were carried out under an atmosphere of nitrogen using standard Schlenk techniques **[31]**. Benzene and chloroform were distilled from CaH_2, stored over 3Å molecular sieves and deaerated by purging with nitrogen immediately before use. Thin-layer chromatography was performed using Merck glass plates pre-coated with F_{254} silica gel 60; compounds were visualized by UV and/or with *p*-anisaldehyde stain solution. Flash chromatography was performed using EM Science silica gel 60, following the procedure of Still [32], with the solvent mixtures indicated. Melting points were measured on a Thomas-Hoover Capillary Melting Point Apparatus, and are uncorrected.

Reagents

All reagents were purchased from commercial suppliers, and used as received, unless indicated otherwise. Dioxane was purchased from Aldrich in Sure-Seal bottles.

Spectroscopic Methods

NMR spectra were measured on Nicolet NT 270 and Varian Mercury 300 MHz instruments at the Georgetown NMR Facility; chemical shifts are reported in units of parts per million relative to Me_4Si. All spectra are recorded in $CDCl_3$. Significant ^1H NMR data are tabulated in the following order: multiplicity (s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet), coupling constants in Hertz, and number of protons. ^{13}C NMR spectra were recorded at frequencies of 67.9 and 75.6 MHz. IR spectra were measured on a MIDAC Corp. or a Mattson Galaxy 2020 Series FTIR, as neat films; absorption bands are reported in cm^{-1}. Low- resolution mass spectra were measured on a Fisons Instruments MD 800 quadrupole mass spectrometer, with 70 ev electron ionization, and a GC 8000 Series gas chromatograph inlet, using a J & W Scientific DB-5MS column of 15 m length, 0.25 mm i.d. and 0.25 μm film thickness. Mass spectra data are given as m/e, with the relative peak height following in parentheses.

Compound Characterization

All new compounds were characterized by ^1H NMR, IR and ^{13}C NMR spectroscopies. Fast atom bombardment mass spectra (FABMS) were recorded at the University of Maryland College Park of Mass Spectrometry Facility. Literature references are given for all known compounds, with the exception of those that are commercially available; all known compounds were identified by ^1H NMR spectroscopy.

Preparation and Characterization of Compounds (Scheme 1)

Step 1: Synthesis of Dehydroprogesterone. *p*-Toluenesulfonic acid monohydrate (11.0 g, 63.9 mmol) was dehydrated in freshly distilled benzene (320 mL) via azeotropic refluxing employing a Dean-Stark trap. After 1 h, cooled the solution for 0.5 h, and progesterone (5.0 g, 15.9 mmol) and 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (4.6 g, 20.3 mmol) were added. The olive mixture was refluxed for 3 hrs, and then was filtered through a pad of Celite. The filtrate was washed with sat. NaCl (5 x 20 mL), followed by 1% NaOH solution until it gave clear solution, and then dried over $MgSO_4$. The solvent was removed under reduced pressure and purified by chromatography.

Step 2: Synthesis of 7 -[4'-(aminophenyl)thio]-pregna-4-ene-3,20-dione (PgA1), 7 -[4'-(aminophenyl)thio]-pregna-4-ene-3,20-dione (PgA37) or 7 -[4'-(aminophenyl)thio]-pregna-4-ene-3,20-dione (PgA39). Dehydroprogesterone (1.65 g, 5.28 mmol), NaOH (pellet, 116 mg, 2.9 mmol), and 4-aminothiophenol (for PgA1), or 3-aminothiophenol (for PgA37) or 2-aminothiophenol (for PgA39; each, 1.32 g, 10.56 mmol) were placed in a Schlenk tube, which was purged with a constant flow of N_2 (g). Deoxygenated anhydrous dioxane (25 mL) was added and heated at 74°C for 6 days. The mixture was then concentrated under reduced pressure, purified by chromatography.

Step 3: Synthesis of additional progesterone analogs. A suspension of PgA1, or PgA37, or PgA39, in degassed chloroform was treated with the appropriate isocyanates under N_2. The mixture was stirred for 12 hrs, and then chromatographed directly on silica gel to afford the corresponding ureas as oil. The resulting oil was stirred in ether until white powder came out.

RESULTS

OBJECTIVE 1: *IN VIVO* EVALUATION OF PGA4'S TOXICITY AND MDR1-REVERSING ACTIVITY

TASK 1: FORMULATION

Like most known MDR1-reversing agents, PgA4 appears to have a limited solubility in water (Table 1). To optimize PgA4 formulation and maximize the dose that can be delivered *in vivo*, we evaluated PgA4 solubility in different solvents. Ethanol, DMSO, propylene glycol and glycerin are all solvents used in classical formulations. However, because of their local or systemic toxicity they must be used in combination with other solvents, *e.g.*, the use of ethanol at concentrations higher than 10% is contraindicated, while DMSO's LD_{50} (acute toxicity following *iv* administration) in mice is about 6 g/Kg [33]. Cyclodextrins appear to increase effectively the solubility of steroids and other lipophilic drugs by the formation of inclusion complexes. These compounds are relatively non-toxic: 2-hydroxypropyl-β-cyclodextrin 10 g/Kg *i.p.* caused no death among four mice [34].

In preliminary evaluations, we characterized PgA4 UV profile. PgA4's Specific Absorption Coefficient (E_1^1) at 245 nm wavelength is 376.1+/- 4.3 SE (average of 3 determinations).

We defined PgA4 solubility in water, ethanol, DMSO, propylene glycol, glycerol and 20% 2-hydroxypropyl-β-cyclodextrin (HPCD). Our results (reported in Table 1) suggest maximum solubility in ethanol and DMSO, with good solubility in propylene glycol. Solubility in HPCD, though more than 10 times lower than in ethanol, appeared the most promising, as HPCD represents a complete formulation. In a direct comparison, 20% HPCD appeared superior to a classical formulation (10% ethanol; 40% propylene glycol), the solubility of PgA4 being respectively 2.4 and 0.4 mg/ml in the two vehicles.

To exclude the possibility that the interaction with the cyclodextrin may affect PgA's action on Pgp, we compared the ability of PgA4 prepared from HPCD and ethanol stock solutions to increase

doxorubicin in LCC6/MDR1 cells *in vitro*. By demonstrating that the biological activity of drug preparations from the two stocks is equivalent (Fig. 1), the results confirm that HPCD does not alter PgA4's interaction with Pgp.

Based on the above results, we selected 20% HPCD as the PgA4 formulation vehicle for all subsequent *in vivo* studies.

TASK 2: SETTING UP AN ASSAY FOR THE EVALUATION OF *IN VIVO* MDR1 REVERSING ACTIVITY

Doxorubicin accumulation assay for the preliminary evaluation of *in vivo* MDR1-reversing activity.

In vitro evaluations have confirmed that MDR1 expression confer MDA435/LCC6^{MDR1} cells the ability to maintain lower intracellular levels of the anticancer substrates doxorubicin as compared to the parental cell line MDA435/LCC6 (Fig. 2). We have observed a 6.5-fold difference (mean of 5 separate determinations, +/- 0.7 SE) in intracellular accumulation of the drug under after treating cells with doxorubicin 4 μM for 3 hours in *in vitro* conditions. This difference is completely reversed by treatment with MDR1-reversing agents (see below, and Fig. 6).

We considered the use of a substrate accumulation assay also for our preliminary evaluations of PgA4 *in vivo* MDR1-reversing activity. With respect to other classical endpoints, the effect of test drugs on the accumulation of an anticancer drug in Pgp-positive human xenografts appears to have some theoretical advantages: (1) experiments are shorter, and consequently less expensive; (2) increased toxicity and possible animal loss due to pharmacokinetic interactions between chemosensitizer and anticancer drug represent less of a concern, as the animals are sacrificed a short time (24 hours) after anticancer drug treatment (much before the appearance of bone marrow toxicity); (3) anticancer drug cell accumulation *in vivo* represents a more humane endpoint with respect to survival experiments; (4) anticancer substrate accumulation is closely related to Pgp's mechanism of action.

Doxorubicin represents a convenient first choice as the tracer substrate in substrate accumulation assays, because it can be easily quantified by fluorimetry. Moreover, doxorubicin is a critical anticancer drug in breast cancer treatment. Using an ascites model reduces factors related to a solid tumor's 3-dimensionality, such as reduced blood perfusion and hypoxia in the center of the tumor, increased intratumor pressure, which complicate the interpretation of results. Ascites are obtained by inoculating 1 x 10^6 cancer cells (either the Pgp-positive MDA435/LCC6^{MDR1} or Pgp-negative MDA435/LCC6 cells) in the peritoneal cavity of NCr *nu/nu* mice. Ascites become evident, on average, after about 3 weeks after cell inoculation, and are represented by cancer cells floating in extracellular ascitic fluid.

For the doxorubicin accumulation assays, mice are treated as soon as enough ascitic fluid is judged to be available for the analysis of substrate/tracer concentrations. At the defined time after treatment with doxorubicin, mice are sacrificed, and ascites collected. Immediately after collection, the ascites cells are separated from the extracellular ascitic fluid by centrifugation. The main experimental

endpoint can be represented by doxorubicin accumulation in the ascites cell pellet. Alternatively, the evaluation of the ratio of doxorubicin concentrations in the ascites pellet and supernatant provide an advantage in helping to reduce the variability due to differences in doxorubicin pharmacokinetics between different mice. In this assay, the parallel use of the parental MDR1-negative cells as control can help discriminate modifications in doxorubicin accumulation due to pharmacokinetic modulation rather than to interaction with the Pgp expressed on cancer cells (as these are reflected in changes in intracellular levels of doxorubicin, independent of Pgp status).

Assay validation. We first aimed to validate the doxorubicin accumulation endpoint and to define an optimal time for the collection of ascites following *in vivo i.v.* inoculation of doxorubicin. At this aim, at ascites maturity, Ncr n*u/nu* mice were treated with 12 mg/Kg of doxorubicin. Mice were sacrificed 1, 3 and 12 hours after doxorubicin administration and the ascites collected. The results of this evaluation validated the ascites doxorubicin accumulation model by confirming the differential accumulation of doxorubicin in MDA435/LCC6 and MDA435/LCC6^{MDR1} cells. The difference is not evident at 1 hour, but becomes apparent at 6 hours (2.4-fold), and is maximal at 24 hours (10.8-fold) (Fig. 3). So, these results suggest that, among the time points tested, the difference in doxorubicin accumulation is best appreciated 24 hours after doxorubicin *i.v.* administration.

TASK 3: EVALUATION OF *IN VIVO* TOXICITY

Before we could proceed to test the *in vivo* MDR1-reversing activity of PgA4, we needed to define the toxicity of the PgA4 treatment regimen to be used in combination with doxorubicin.

In one experiment, we used 4 NCr *nu/nu* female athymic nude mice: two mice were treated with PgA4 15 mg/Kg (10 µl/g body weight of a solution of PgA4 1.5 mg/ml in HPCD), twice a day for 3 days; two more mice were treated with an equivalent amount of vehicle (20% HPCD), following the same schedule of administration. This treatment showed no acute toxicity either in terms of mortality, altered behavior, or signs of local toxicity. A longer term evaluation showed no significant effect of PgA4 treatment on body weight gain up to 55 days following the completion of treatment (Fig.4).

In a separate evaluation, we examined the effect of PgA4 treatment on hematologic, liver and kidney toxicity. 5 NCr *nu/nu* female athymic nude mice were treated with PgA4, 19 mg/Kg (10 µl/g body weight of a solution of PgA4 1.5 mg/ml in HPCD), twice a day for 3 days. 5 more mice were treated with an equivalent amount of vehicle (20% HPCD). The results show a small, but statistically significant difference in the relative percentage of granulocytes and lymphocytes, but no significant change in the overall white blood cell count (data not shown), nor in other hematological parameters, and markers of kidney and liver function and/or toxicity (Table 2).

In conclusion, task 3 was completed to the best of our ability. In our *in vivo* toxicity experiments no *dose-limiting toxicity* and consequently no *maximum tolerated dose* could be reached. Consequently, no *maximum tolerated dose* could be defined. The *maximum achievable dose* of PgA4 (MAD) (15-20 mg/Kg per inoculation), is defined not by the drug's toxicity (MTD), but by its solubility and by the maximum volume that can be administered humanely to mice.

TASK 4: EVALUATION OF *IN VIVO* MDR1-REVERSING ACTIVITY

Evaluation of PgA4's effect on the toxicity of doxorubicin and taxol.
It is well known that several MDR1-reversing agents also may affect the toxicity of anticancer drugs by altering their pharmacokinetics (possibly, through interaction with Pgp and/or other ABC proteins in normal excretory tissues). Consequently, MDR1-reversal studies based on a survival or a tumor growth endpoint should use equitoxic doses of the anticancer drug in the presence and absence of the MDR1 reversing agent.

We evaluated the effect of the maximal achievable dose of PgA4 on the toxicity of doxorubicin and taxol, as evaluated in terms of inhibition of body weight gain. These two drugs were selected because they appear to be the most effective agents presently available for the treatment of breast cancer.

Effect of PgA4 on doxorubicin toxicity. During a 4-weeks follow-up, we observed no evidence that PgA4 (administered s.c., twice daily for 6 times at a dose of of 28 mg/Kg) affected the effect of doxorubicin (administered *i.v.* at 8 mg/Kg) on the body weight gain of NCr *nu/nu* female athymic nude mice. So, PgA4, administered as described above did not appear to affect the toxicity of doxorubicin.

Effect of PgA4 on taxol toxicity. We evaluated the effect of PgA4 on the *in vivo* toxicity of taxol. For this experiment, taxol was administered *i.v.* at 8 mg/Kg weekly for four weeks. PgA4 was administered *s.c.* at a dose of 15 mg/Kg, 6 times weekly: at the time of taxol treatment, and 48, 36, 24, 12 hours before, and 12 hours after taxol treatment. No difference in body weight gain was observed following treatment with taxol with and w/o PgA4.So, as in the case of doxorubicin, treatment with PgA4 did not affect the *in vivo* toxicity of taxol .

In conclusion, PgA4, at the maximum achievable dose, did not affect the toxicity of doxorubicin and taxol, when these were administered at doses usually effective *in vivo*. Consequently, no modification of the dose of doxorubicin and taxol was required in our subsequent *in vivo* MDR1 reversing studies.

Effect of PgA4 on doxorubicin accumulation in ascites cells
Effect of PgA4 on in vivo doxorubicin accumulation in MDA435/LCC6^{MDR1} ascites cells. The results of an initial experiment, carried out on a limited number of mice per treatment group, confirm that the ratio of intracellular/extracellular concentrations of doxorubicin in LCC6^{MDR1} is lower than in LCC6 ascites cells (these results are statistically significant). More interestingly, these results show that PgA4 treatment increases the ratio of intracellular/extracellular concentrations of doxorubicin, and it does so specifically in Pgp(+) LCC6^{MDR1} ascites (Fig. 5). These data appeared to represent a promising preliminary evidence of PgA4's effectiveness *in vivo*, but the results did not reach statistical significance because of the low number of mice.

A higher number of animals was used in a second experiment. 20 animals were treated with

doxorubicin (12 mg/Kg *i.v.*) and 20 more with doxorubicin +PgA4 (30 mg/Kg per inoculation). Two 10-mice control groups were treated respectively with vehicle only and PgA4 only. The results of this test are represented in Fig. 6 The effect of PgA4 on doxorubicin accumulation in MDA435/LCC6^{MDR1} ascites cells was minimal and the results did not confirm the *in vivo* MDR1-reversing efficacy suggested by the prior test.

Effect of PgA4 on tumor growth inhibition by taxol.
In two separate studies, PgA4 was administered in combination with respectively doxorubicin and taxol. In both these studies we evaluated the effect of PgA4 treatment on the ability of the respective anticancer drug to inhibit the growth of Pgp-positive MDA435/LCC6^{MDR1} cell tumor xenografts.

Effect of PgA4 on tumor growth inhibition by taxol. 4 treatment combinations were compared: *i.* Vehicle only (control); *ii.* PgA4 only; *iii.* taxol only; *iv.* PgA4 + taxol. Unluckily, the results obtained in this test (Fig. 7) are not reliable, as the MDA/LCC6 tumors showed a slower growth and inhibition by taxol was only, counterintuitively, observed for MDA435/LCC6^{MDR1} tumors. Using an *in vitro* functional assay and cells recovered from this experiment's tumors, we have confirmed the tumor's identity and excluded a cell mix-up.

Effect of PgA4 on the survival of mice carrying P388/ADR ascites and treated with doxorubicin
When administered locally (*i.p.*), PgA4 appeared to increase from 11 to 17 days the median survival of mice inoculated with Pgp-positve P388/ADR cells and treated with doxorubicin (Fig. 8). However, systemic administration of PgA4 (*s.c.*, in the interscapular region) did not increase the ability of doxorubicin to increase survival (Fig. 9).

OBJECTIVE 1: CONCLUSIONS
With the exception of some initial promising results, the MDR1 reversing efficacy of PgA4 could not be confirmed *in vivo* in studies where the drug was administered systemically. By contrast, local administration of PgA4 appears to be effective, suggesting that the main reason for PgA4sapparent lack of *in vivo* efficacy may be related to its pharmacokinetic and/or metabolic disposition. We are now setting up the analytical assay that will allow us to investigate the concentration of PgA at the target tissues.

OBJECTIVES 2 AND 3: BRIDGE AND F-RING OPTIMIZATION, *IN VITRO* MDR1-REVERSING ACTIVITY AND TOXICITY

MDR1-REVERSING ACTIVITY

Overall, we have designed and synthesized twenty-four C-7 progesterone analogs. Our aim was to further optimize the MDR1-reversing activity observed with the early derivatives and to characterize structure-activity relationships. Potency was quantified in terms of EC_{50}, *i.e.*, the drug concentration necessary to reduce by 50% the difference in doxorubicin intracellular accumulation between MDA435/LCC6^{MDR1} and the parental MDR1-negative MDA435/LCC6 cells. The general structure of the PgA compounds is summarized in Fig. 10. The results of our activity tests are summarized

in Tables 3a, 3b and 3c in terms of potency relative to the parental compound progesterone without (second column from the right) or with correction (first column from the right, labeled "MDR1-specific") for any non-specific effect of the test compound on the MDR1-negative control cells. We examined the relation between the activity of the PgA compounds and some of their structural features.

Length of the alkyl chain distal to the C7 urea-E ring moiety. The C7 progesterone analog PgA3 (7α[4'-(N-ethylaminoacylaminophenyl)thio]pregna-4-ene-3, 20-dione), which includes in the C7 side chain an ethyl group bound to an aromatic e-ring through a urea group, is about 40-fold more potent than the parental compound progesterone in reversing the MDR1 phenotype (in terms of effect on doxorubicin accumulation in MDA435/LCC6^{MDR1} cells). The length of the alkyl group ("ethyl" in PgA3) may effect the compound's MDR1activity. A propyl group (as in PgA41) in lieu of the ethyl group (as in PgA3) may somewhat increase the activity. However, further elongation of the alkyl chain bound to the urea group appear to be counterproductive, possibly because of problems of steric hindrance: butyl- and hexyl- substituents (as, respectively, in PgA36 and PgA35) appear to decrease the activity with respect to PgA3. However, the data for PgA35, PgA36, and PgA41 need confirmation.

Role of the urea group. An obvious way to evaluate the role of the urea group would be to evaluate the activity of of a C7 progesterone analog deprived of this group. In PgA1 (7α[4'-(aminophenyl) thio] pregna-4-ene-3, 20-dione), the precursor of most of our C7 analogues, a primary amine group substitutes the alkylurea group in the *para* position of the E-ring. Unfortunately, the MDR1 activity of PgA1could not be evaluated because of problems with the compound's solubility. We were, however, able to compare the activity of PgA37, an isomer of PgA1 where the amine group is substituted in *meta* as compared to *para* position, with its ethyl urea derivative PgA38 (isomer of PgA3). The ethylurea derivative appears to be about twice as potent as the parental compound. The comparison, however, is not entirely adequate, because the role of the urea group cannot be discriminated from the role of the distal group length.

Position of the alkylurea substituent on the E ring. Comparison of PgA3 (alkylurea group in *para* position on the E-ring), PgA38 (*meta*) and PgA40 (*ortho*), suggests slightly higher activities when the alkylurea group is substituted in the *meta* position (about 60-fold more potent than progesterone) on the E-ring. A loss of potency was observed for the *ortho* isomer (about 18-fold more potent than progesterone). Advantage conferred by the addition of an aromatic F-ring (PgA13) is lost when this is part of group substituted in the *meta* position of the E-ring.

Role of polarity in the distal C7 side chain. Polarization of the ethyl substituent in PgA3 by chlorination (PgA2) does not appear to obviously alter its ability to modulate doxorubicin accumulation in MDR1 cells.

Role of F-ring and bridge length. The presence of an aromatic F ring (as in PgA13) confers more than a 3-fold increase in MDR1 cell-specific doxorubicin accumulation effect (as compared to PgA3), but only when the ring is directly attached to the urea group. Longer bridges between E and F ring fail to show an obvious (>2-fold) advantage when compared with the effect of compounds

without a F ring. This evidence may suggest that the aromatic F ring in PgA13 is more favorably located for $\pi-\pi$ interaction with the aromatic amino acids on Pgp.

Role of a partial positive or negative charge on the F ring. The increased potency conferred by an aromatic F ring (in PgA13), appears to be lost following its substitution with an electron-withdrawing p-trifluoromethyl- group (PgA28), suggesting that acquisition of a partial positive charge negatively affects the ability of the F ring to interact with Pgp. Also the addition of other electron-withdrawing or electron-donating groups on the F ring (as in the PgA13 analogs PgA20, PgA30, PgA31, PgA32, and PgA34 appears to decrease the MDR1 reversing effect. However, a simple steric hindrance effect of the substituent on the F ring cannot be completely ruled out.

Role of a third aromatic ring ("G"). Addition of a third aromatic ring, distal to the "F" ring, as in PgA29, appears to inhibit the MDR1 reversing potency, possibly because of steric hindrance.

IN VITRO ACTIVITY/TOXICITY RATIO

We have evaluated the *in vitro* toxicity of some of our test compounds and compared this with the toxicity of the reference MDR1 reversing agents verapamil and cyclosporin A. The cytotoxicity of the different compounds is reported in Table 4 in terms of IC_{50}, the concentration inhibiting 50% cell growth. For a more adequate comparison of drug efficacies, table also provides an estimate of "toxicity-corrected" efficacy, in terms of the ratio of IC_{50} (for toxicity) and EC_{50} (for MDR1 reversing activity) values for each drug. For PgA3 and PgA4, a 50% level of growth inhibition could not be reached at the maximum concentration that, for limits of solubility, could be obtained *in vitro*. So, for these compounds the reported ratios represent only minimum estimates. The results show that, while the MDR1-reversing potency of PgA3 and PgA4 is about 4-times higher than that of verapamil and comparable to that of cyclosporin A, the toxicity-corrected *in vitro* MDR1 reversing efficacy of these compounds far exceeds cyclosporin A's, an agent with a high level of *in vitro* toxicity.

OBJECTIVES 2 AND 3: CONCLUSIONS

C-7 substitution of the progesterone molecule with a bulky moiety has allowed us to obtain to obtain with reduced or no hormonal (progestational, glucorticoid) activity and MDR1 activity increased up to 170-fold as compared to the parental compound. The most potent of the analogs (PgA13) is 2 and 15 fold more potent than respectively cyclosporin A and verapamil. There appears to be a correlation between the activity of these compounds and some of their structural features, such as in particular the location of the distal moiety substitution on the E ring, length of the distal moiety and presence of a second aromatic ring (F).

OBJECTIVE 4: MECHANISM OF MDR1-REVERSAL BY PGA COMPOUNDS

Evidence from the literature supports the hypothesis that PgA compounds may reverse the MDR1 phenotype by competing with anticancer substrates for the Pgp binding site(s). Steroidal compounds have been shown both to reverse the MDR1 phenotype and to be actively transported by Pgp, the former activity appearing directly and the latter inversely proportional to hydrophobicity [7, 25, 35-37]. Moreover, photoaffinity labeling studies demonstrate direct binding of steroids to Pgp [36, 38].

Our *in vitro* toxicity studies (see above) appear to confirm the hypothesis. In fact, by showing a relative resistance of MDR1-expressing cells to PgA2 and PgA5 (about 2-3 fold), the results of the *in vitro* toxicity tests also suggest that these C7 progesterone analogs are substrates for Pgp (Table 4).

It is unclear whether Pgp binds its substrates at one or more binding sites. Studies using classical membrane binding assays and the analysis of dissociation rates have shown that some substrates, though interacting "competitively", might actually bind to distinct, though allosterically connected, sites [10, 11]. It may be speculated that the PgA binding site(s) may, either partially or totally, overlap with those of some anticancer substrates and be only allosterically connected to others. It is also possible that MDR1 susbtrates bind to shallow sites on an extended binding surface and that partial overlapping may be affected by Pgp conformation as determined by the specific membrane environment.

To address these questions, we have been developing an experimental model for the on-line liquid chromatographic determination of substrate-binding affinities and substrate-substrate interaction at Pgp sites. This model is based on the immobilization of Pgp in a chromatographic column stationary phase.

Initial characterization of the Pgp column

An evaluation of protein content showed that, for one milliliter of bed volume, about 170 mg proteins were immobilized on the IAM column and about 10 mg proteins were immobilized on Superdex 200 column. The chromatographic results obtained with the Pgp-IAM column or Pgp-Superdex 200 column indicated that the binding activity of Pgp was retained after immobilization. For example, [^{3}H]-vinblastine was retarded on a Pgp-IAM column (0.5 x 0.8 cm) and the retention volume was 13.3 ml at the concentration of 1 nM (profile A in Fig. 11) at flow rate: 0.4 ml/min. When a displacer ligand, doxorubicin (200 nM), was included in the mobile phase, the retention volume of 1 nM [^{3}H]-VBL was decreased from 13.3 ml to 6.5 ml (profile B in Fig.11). This indicated that the retardation was partially due to the specific binding to saturable binding sites of Pgp. The obtained K_d value for [^{3}H]-vinblastine determined in this technique is 19 ± 20 nM, that is consistent with the reported value, 36 ± 55 nM (mean ± SD) [39]. These results have been published [29] and indicate that Pgp-based chromatographic stationary phase can be used for the investigation of Pgp-substrate interactions.

Further characterization of ligand binding on the Pgp column

After the initial characterization and validation, we have used this "Pgp" column model in frontal and zonal chromatographic studies, to further investigate the binding of Pgp substrates such as vinblastine, doxorubicin, verapamil and cyclosporin A. The relative dissociation constants in the absence of ATP were calculated to be 23.5 ± 7.8 nM for vinblastine, 15.0 ± 3.2 μM for doxorubicin, 54.2 ± 4.7 μM for verapamil, and 97.9 ± 19.4 nM. Addition of 3 mM ATP increased the affinity of [^{3}H] vinblastine and [^{3}H] verapamil, and decreased the affinity of [^{3}H] cyclosporin A. Moreover, we observed that, in the absence of ATP, there was no retention of [^{3}H] cyclosporin A on the column, but that retention became apparent in the presence of vinblastine. When [^{3}H]vinblastine was used

as the marker ligand, results showed that its affinity to the column was decreased in the presence of cyclosporin A. Taken together, these and other results from these studies demonstrated that competitive interactions occurred between doxorubicin and vinblastine, and cooperative allosteric interactions occurred between vinblastine and cyclosporin A, and ATP and CsA, and anticooperative allosteric interactions occurred between ATP and vinblastine and verapamil. The results of this study have been published and they can be seen in detail in the attached reprint [30].

OBJECTIVE 4: CONCLUSIONS

Present evidence (including our evaluation of PgA differential toxicity in the presence and absence of Pgp) appears to support competition for Pgp substrate binding sites as the mechanism for PgA compounds MDR1-reversing activity. However, we have not yet completed our competition studies to prove this hypothesis. The studies reported above indicated that the Pgp affinity chromatography model can be used for the study of drug-drug binding interactions on Pgp, but we have still to use this model for the evaluation of the interaction of our PgA compounds with Pgp and the other substrates. A critical collaborator (Dr. Irving Wainer) moved to a new position, and he is now setting up a new research lab at the National Institutes of Health. By mutual agreement, evaluation of the PgA compounds represents one of the top priorities as soon as the all the needed equipment is in place in the new laboratory. The evaluation of PgA compounds' interaction with Pgp will be part of a wider study aimed at functionally define Pgp binding sites and the substrates that share them.

KEY RESEARCH ACCOMPLISHMENTS

- Initial evaluation of one of the Progesterone analogues *in vivo* MDR1 reversing activity
- Optimization of *in vitro* MDR1-reversing activity: the most potent analogue (PgA13) is now more than 150-fold more potent than the parental compound progesterone, more than 15-fold more potent than verapamil, and about 3-fold more potent than cyclosporin A).
- Characterization of the *in vitro* pharmacological activity/toxicity ratio of some of the analogues: this ratio is at least 20 times better for the progesterone analogue PgA4 than for the standard reference drug cyclosporin A.
- Evaluation of structure-activity relationships of the C7-moiety of C7-progesterone analogues: length, role of a second and third aromatic ring, polarity, position of side chain on E-ring.

REPORTABLE OUTCOMES

Papers

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Other relevant manuscripts in preparation

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CONCLUSIONS

Using a substrate (doxorubicin) accumulation endpoint, we have tested the *in vivo* MDR1 reversing activity of PgA4, the most potent *in vitro* MDR1 inhibitor among our early compounds. Though the results of an initial test were suggestive of an *in vivo* effect, differences were not statistically significant. Also a second *in vivo* test failed to demonstrate a statistically significant effect. In future experiments, we will further test the *in vivo* activity of PgA4, using alternative endpoints (solid tumor growth, survival), and cell models (P388/ADR cells).

An expanded panel of C7-progesterone analogs has allowed us to further investigate the relation between the MDR1 reversing activity and the structure of our C7-progestone analogs. The structural features that we have considered include: the length of the C7 moiety (distal to the urea group), the presence of a urea group, the position of the alkylurea substituent on the E ring, the polarity in the distal C7 side chain, the presence of a second and third aromatic ring in the C7 moiety, polarity of the F ring and length of the bridge between rings, and hydrophobicity. Our best compound (PgA13) is more than 150-fold potent than the parental compound progesterone, 10-fold more potent than verapamil and almost 3-fold more potent than cyclosporin.

Not only are some of the C7-progesterone analogs equally or more potent than the classical MDR1 reversing cyclosporin A but, at least *in vitro*, some of then appear much less toxic (20-fold or more) at concentrations which are equiactive on the MDR1 phenotype.

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APPENDICES

Scheme 1. Synthesis of PgA compounds

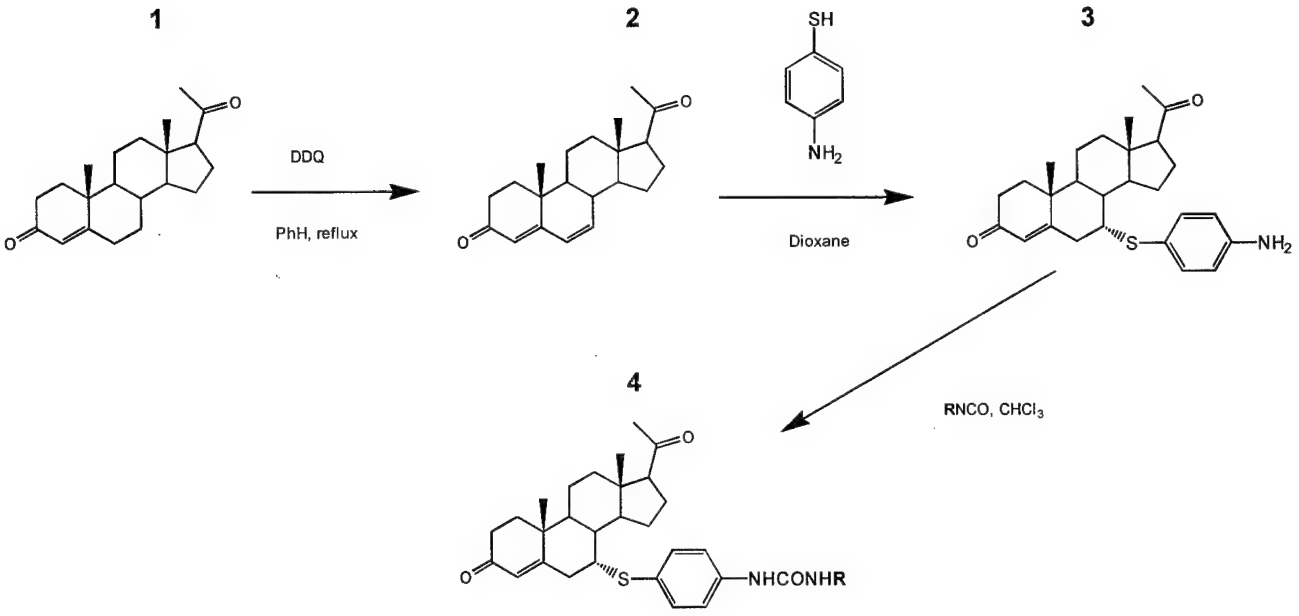


Table 1. Solubility of PgA4 in different solvents

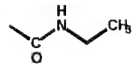
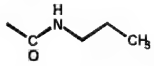
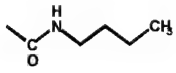
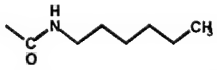
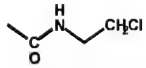
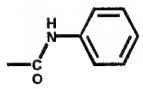
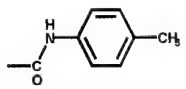
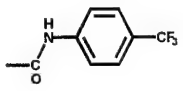
Solvent	*Solubility (mg/ml)*
dH_2O	0.00179
Ethanol	30.4
DMSO	27.216
Propylene glycol	13.371
Glycerin	0.674
20% 2-hydroxypropyl-β-cyclodextrin (HPCD)	2.48

Table 2. Effect of PgA4 on some hematochemical parameters

Parameter (Units)	Treatment		Significance of difference
	Vehicle	PgA4	
Bilirubin, total (mg/dL)	0.10 ± 0	0.14 ± 0.04	P > 0.05
Alkal. Phosphatase (U/L)	247.4 ± 35.4	237.6 ± 33.6	P > 0.05
SGOT (U/L)	123.2 ± 14.7	292.0 ± 162.7	P > 0.05
SGPT (U/L)	75.2 ± 14.3	66.4 ± 9.5	P > 0.05
BUN (mg/dL)	31.8 ± 0.86	31.0 ± 0.84	P > 0.05
Creatinine (mg/dL)	0.46 ± 0.09	0.34 ± 0.04	P > 0.05
Hemoglobin (g/dL)	13.3 ± 0.16	13.5 ± 0.35	P > 0.05
Hematocrit (%)	40.8 ± 1.58	38.3 ± 1.10	
White blood cells (x $10^3/mm^3$)	3.82 ± 0.39	3.13 ± 0.43	P > 0.05
Red blood cells (x $10^6/mm^3$)	8.38 ± 0.05	8.50 ± 0.27	P > 0.05
MCV (fL)	48.8 ± 2.10	45.0 ± 0	P > 0.05
MCH (pg)	15.9 ± 0.28	15.9 ± 0.21	P > 0.05
MCHC (g/dL)	32.8 ± 0.87	35.3 ± 0.52	P > 0.05
Platelet count	989.5 ± 9.5	996.0 ± 3.0	P > 0.05
% Polys.	62.0 ± 2.61	75.0 ± 2.31	P < 0.05
% Bands	0	1.0 ± 1.0	P > 0.05
% Lymph.	37.0 ± 3.34	22.3 ± 3.53	P < 0.05
% Monos.	0.50 ± 0.50	1.67 ± 1.67	P > 0.05
% Eos.	0.50 ± 0.50	0	P > 0.05
% Bas.	0	0	NA

Table 3a. MDR1-reversing potency of different C-7 progesterone analogs, relative to progesterone

PgA: Series I

Analog	*"R" Function*	*MDR1-reversing potency, relative to progesterone*	
		LCC6/MDR1 cells	*MDR1-specific*
PgA3		*31.3*	*42.7*
PgA41		*61.6*	*66.9*
PgA36		*17.1*	*15.5*
PgA35		*26.7*	*27.1*
PgA2		*40.5*	*60.2*
PgA13		*104.8*	*166.7*
PgA20		*90.1*	*108.0*
PgA28		*26.8*	*40.2*

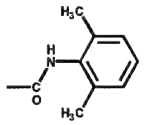
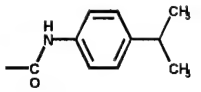
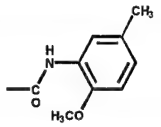
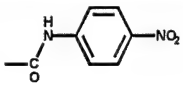
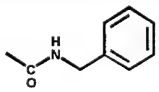
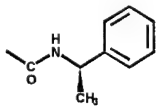
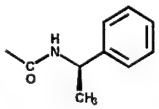
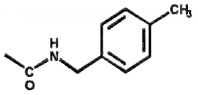
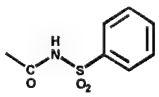
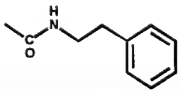
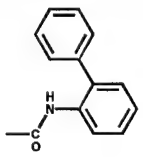
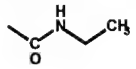
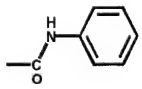
PgA30		***24.2***	***25.6***
PgA31		***22.6***	***31.1***
PgA32		***42.9***	***47.9***
PgA34		***30.6***	***39.1***
PgA8		***35.3***	***40.1***
PgA4(+)		***37.2***	***44.8***
PgA4(-)		***28.3***	***23.8***
PgA11		***32.8***	***30.5***
PgA5		***1.5***	***1.1***
PgA12		***49.2***	***50.8***
PgA29		***8.5***	***16.4***

Table 3b. MDR1-reversing potency of different C-7 progesterone analogs, relative to progesterone

PgA: Series II

Analog	*"R" Function*	*Potency, relative to progesterone*	
		LCC6/MDR1 cells	*MDR1-specific*
PgA37	*H*	*27.8*	*29.5*
PgA38		*54.5*	*69.4*
PgA43		*63.4*	*67.6*

PgA: Series III

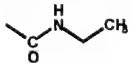
Analog	*"R" Function*	*Potency, relative to progesterone*	
		LCC6/MDR1 cells	*MDR1-specific*
PgA39	*H*	*20.4*	*16.9*
PgA40		*16.7*	*17.7*

Table 3c. MDR1-reversing potency of reference standard agents, relative to progesterone.

Analog	*"R" Function*	*Potency, relative to progesterone*	
		LCC6/MDR1 cells	*MDR1-specific*
Cyclosporin A		*41.9*	*60.6*
Verapamil		*9.2*	*10.2*

Table 4. *In vitro* toxicity and MDR1-activity/ toxicity ratio of C-7 progesterone analogs

Drug	*IC_{50} in MDR1- cells (μM)*	*IC_{50} in MDR1+ cells (μM)*	*Relative resistance in MDR1+ cells*	*IC_{50}/EC_{50}**
Progesterone	*35.35*	*44.69*	*1.26*	*1.1*
PgA2	*3.33*	*9.77*	*2.93*	*13.9*
PgA3	*> 20*	*> 20*	*--*	*> 20.0*
PgA4	*> 20*	*> 20*	*--*	*> 21.2*
PgA5	*20.45*	*38.32*	*1.87*	*1.0*
Cyclosporin A	*0.49*	*0.85*	*1.73*	*1.2*
Verapamil	*65.81*	*62.20*	*0.95*	*15.1*

** IC50 in MDR1-positive cells / MDR1-specific EC_{50}*

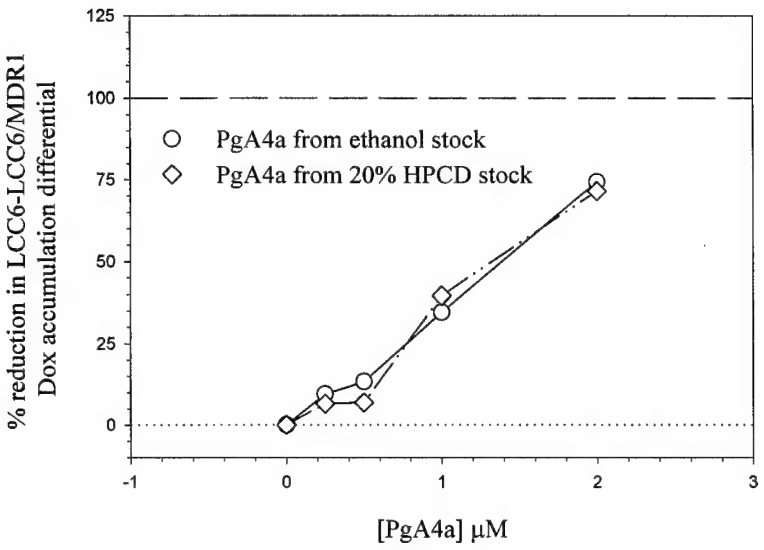


Fig. 1. Effect of PgA4 from ethanol and HPCD stock solutions on doxorubicin accumulation accumulation differential in LCC6/MDR1 cells. Long dashed lines: doxorubicin accumulation in untreated LCC6 cells (defined as 100% of the differential). Medium dashed lines: doxorubicin accumulation in untreated LCC6/MDR1 cells (defined as 0% of the differential).

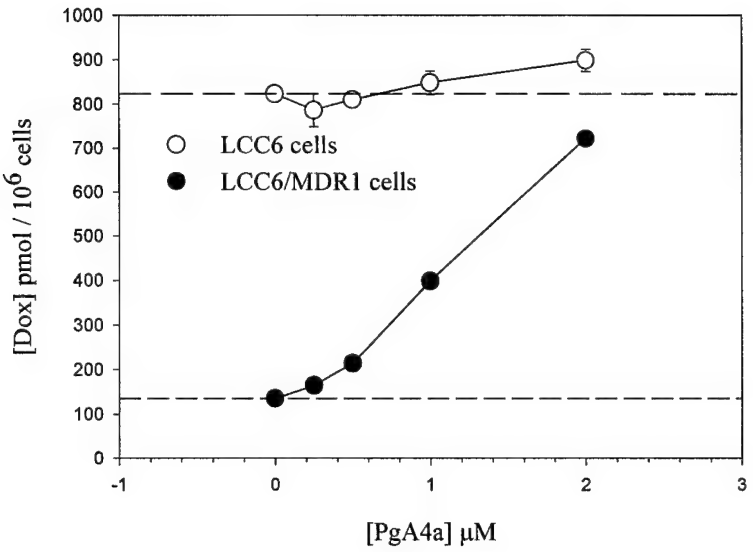


Figure 2. Doxorubicin accumulation in LCC6 and LCC6/MDR1 cells: effect of PgA4 treatment. Long dashed lines: doxorubicin accumulation in untreated LCC6 cells. Medium dashed lines: doxorubicin accumulation in untreated LCC6/MDR1 cells

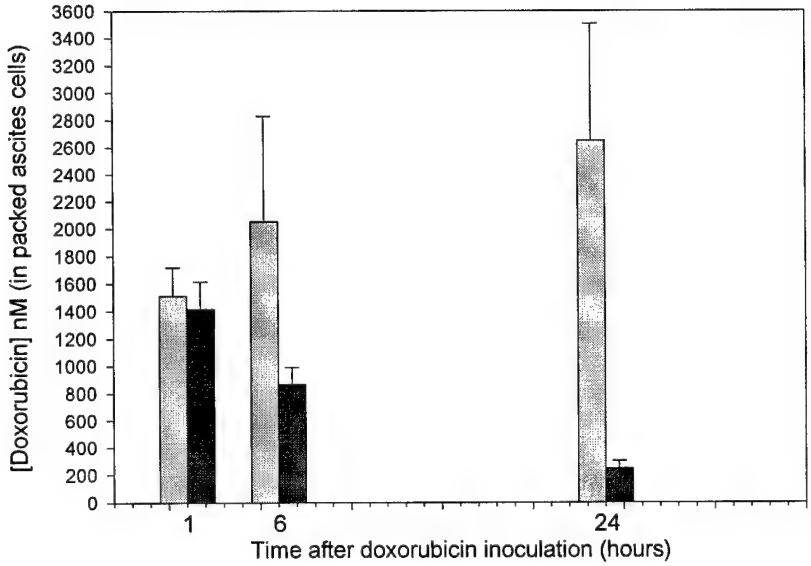


Fig. 3. Time dependence of doxorubicin accumulation in LCC6 and LCC6/MDR1 ascites cells *in vivo*

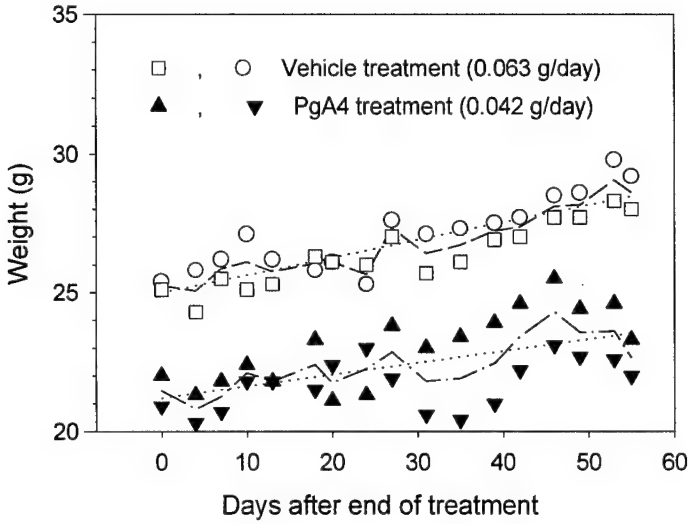


Fig. 4. Weight gain of Ncr *nu/nu* mice following treatment with PgA4. Mice were treated with PgA4 15.4 mg/Kg, twice per day, for 3 days. Each symbol represents values relative to a single mouse Dashed lines represent average values per treatment group.

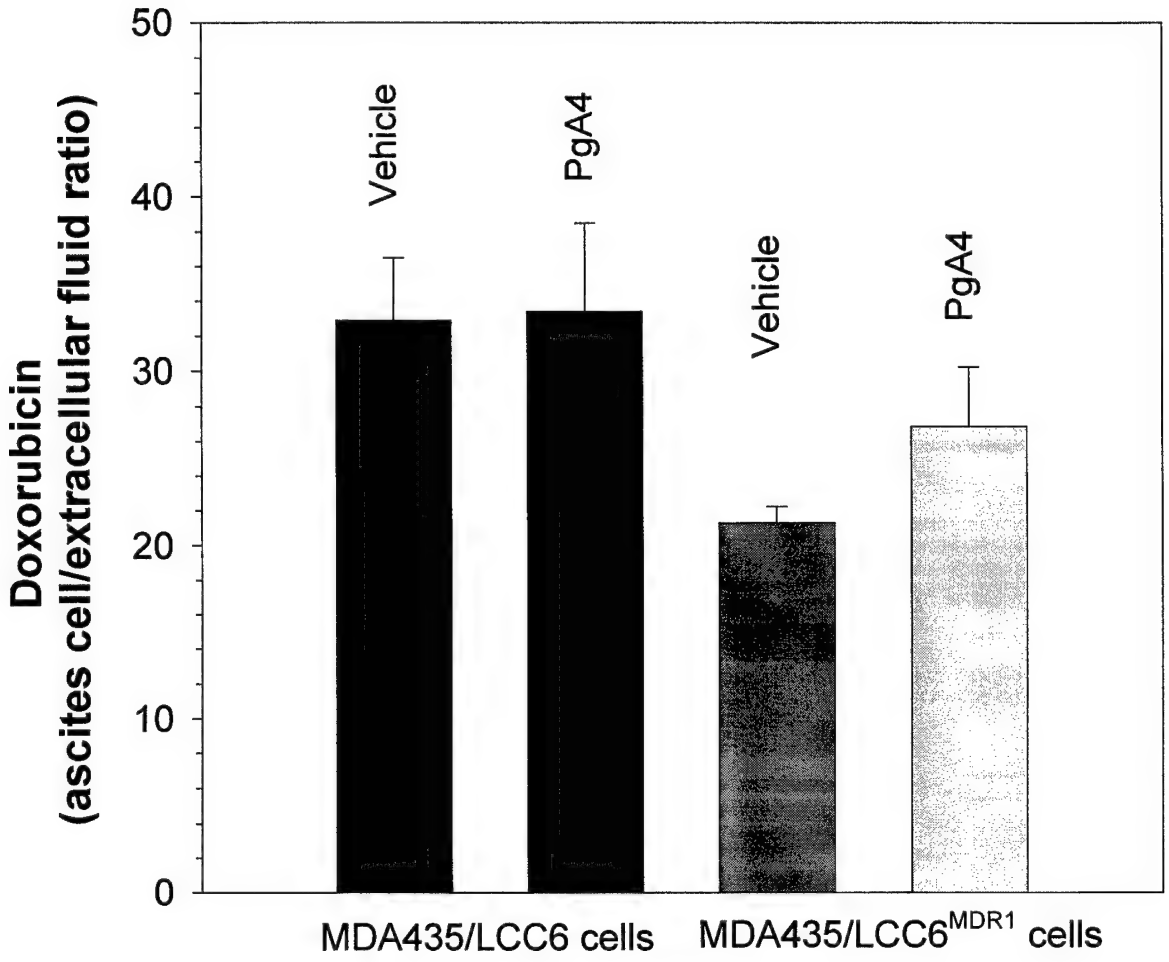


Fig. 5. *In vivo* effect of PgA4 treatment on the ratio of the intracellular/ extracellular concentration of doxorubicin in MDA435/LCC6 and MDA435/LCC6^{MDR1} ascites cells.

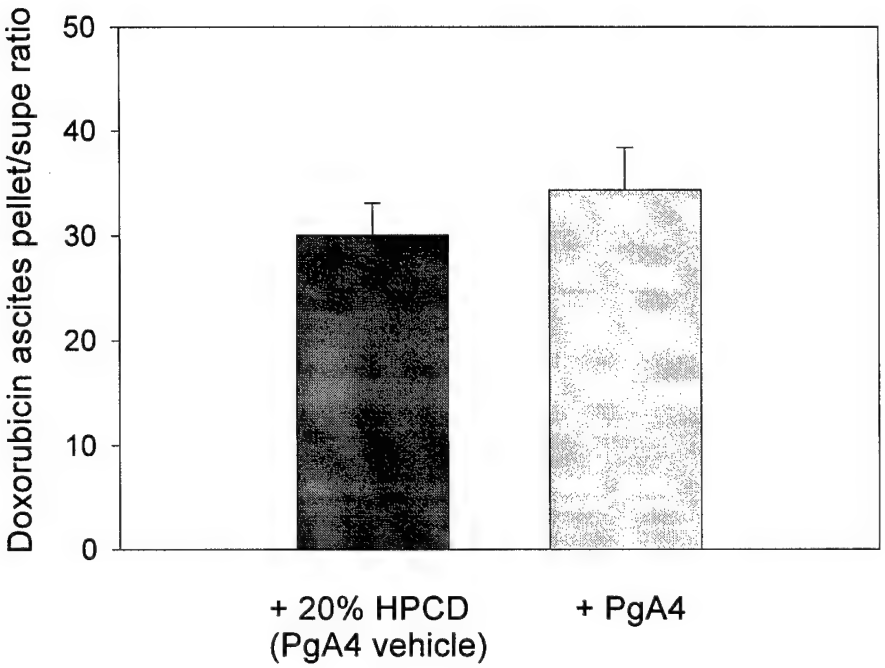


Fig. 6. *In vivo* effect of PgA4 treatment on the ratio of the intracellular/ extracellular concentration of doxorubicin in LCC6/MDR1 ascites cells

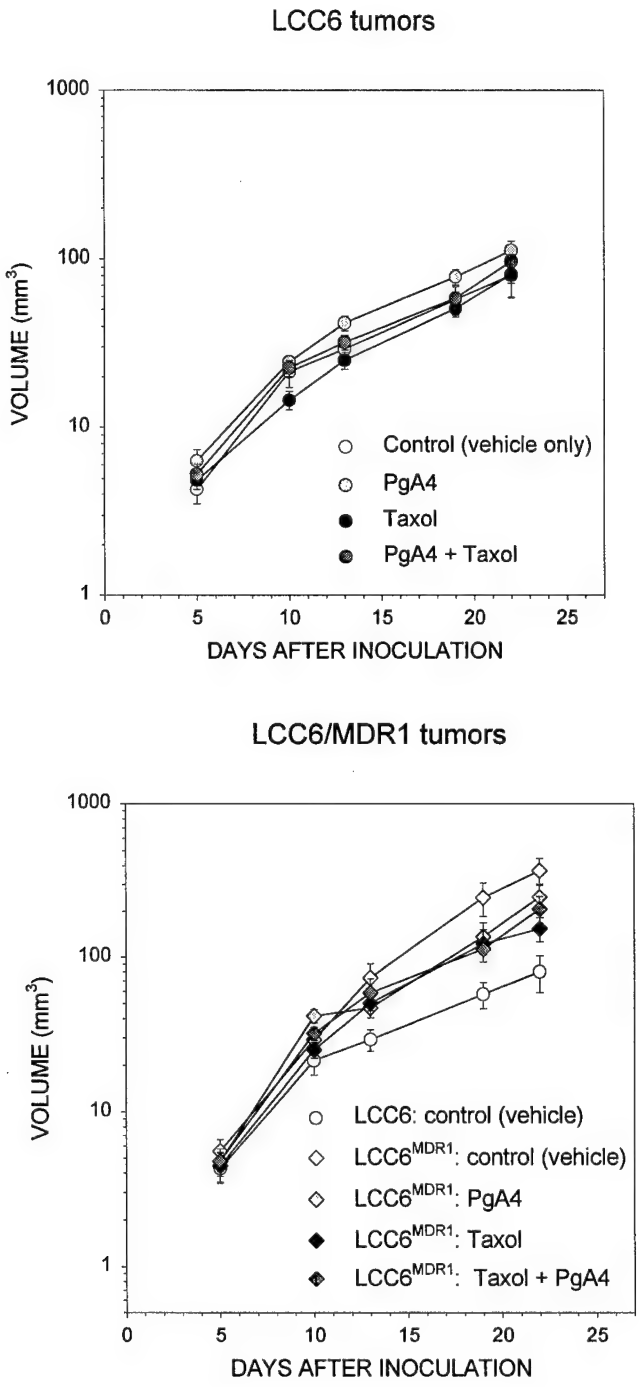


Fig. 7. Effect of treatment with PgA4 and taxol on the growth of MDA435/LCC6 (top panel) and MDA435/LCC6^{MDR1} (bottom panel) tumors

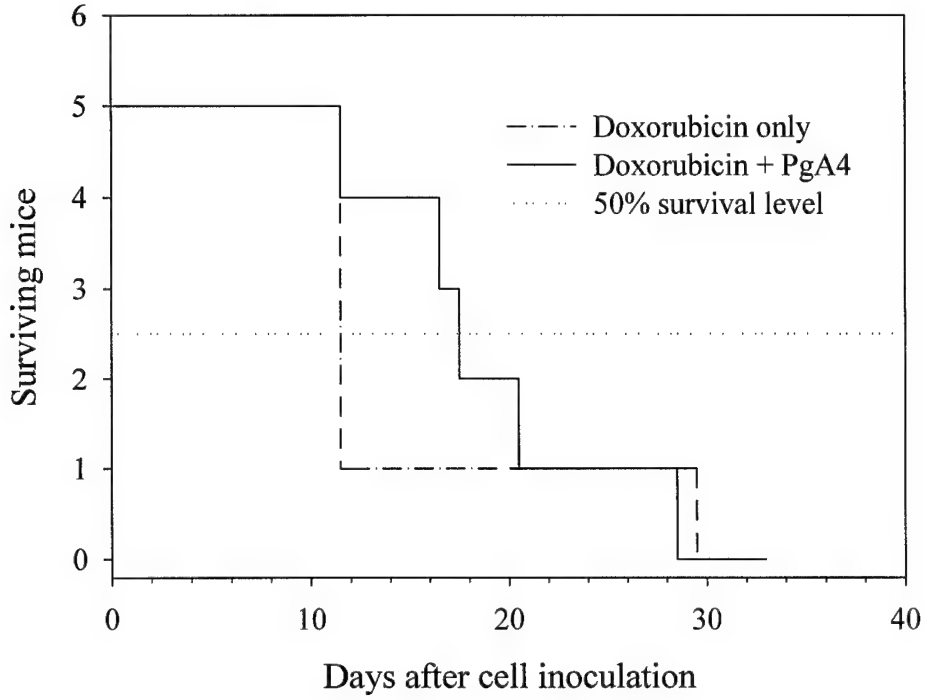


Fig. 8. Effect of PgA4, administered locally (*i.p.*), on the survival of DBA/2 mice inoculated *i.p.* with either Pgp-negative P388 or Pgp-positive P388/ADR cells and treated with doxorubicin.

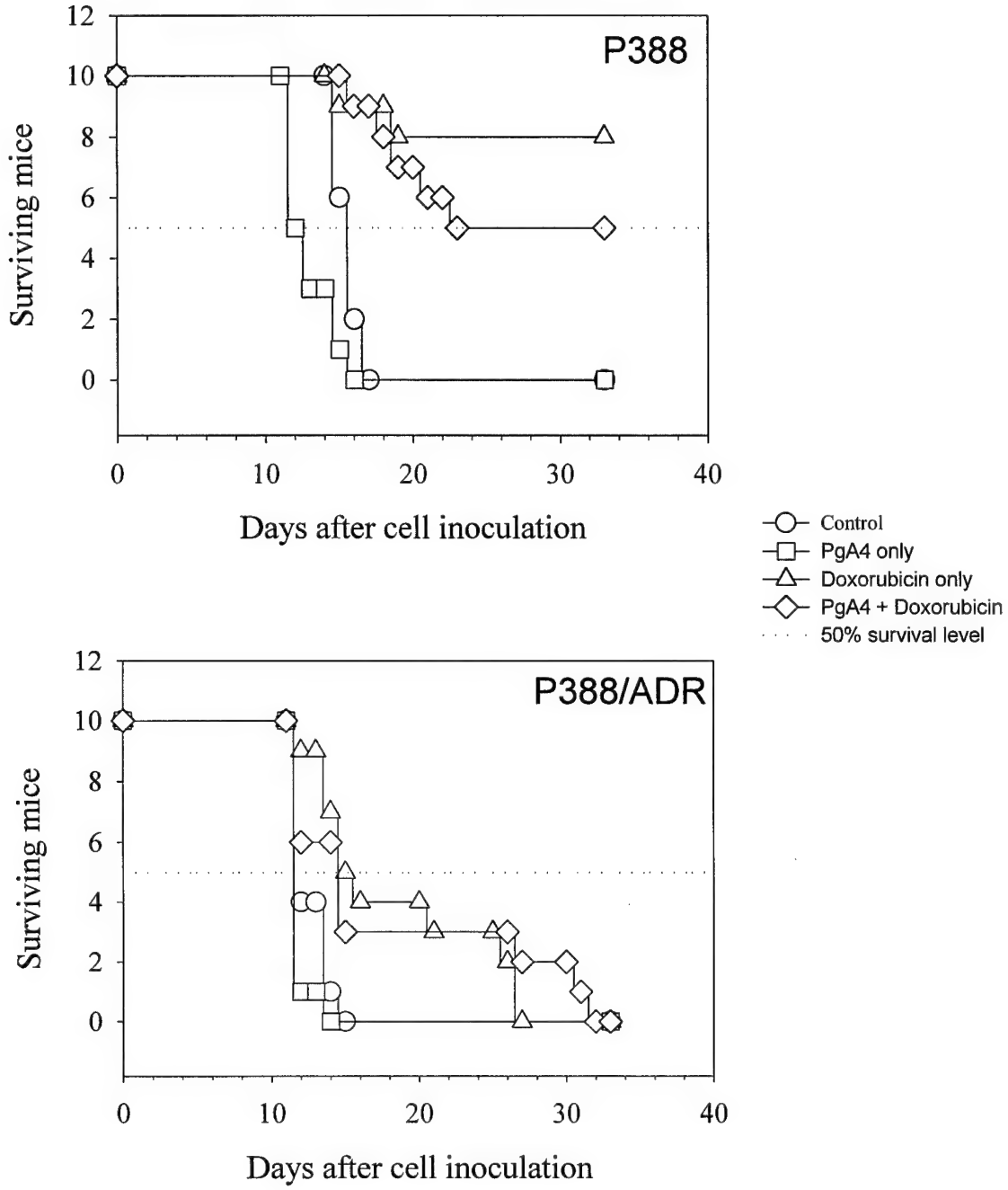
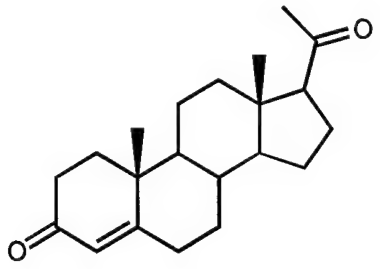
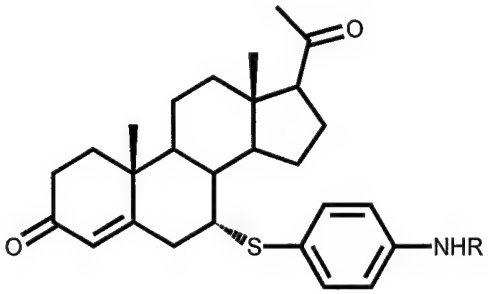


Fig. 9. Effect of PgA4, administered systemically (s.c., in the interscapular region) on the survival of DBA/2 mice inoculated *i.p.* with either Pgp-negative P388 or Pgp-positive P388/ADR cells and treated with doxorubicin.

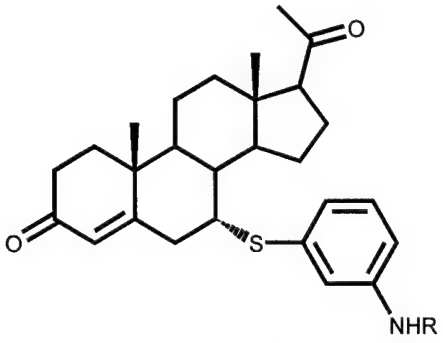
Progesterone



PgA: Series I



PgA: Series II



PgA: Series III

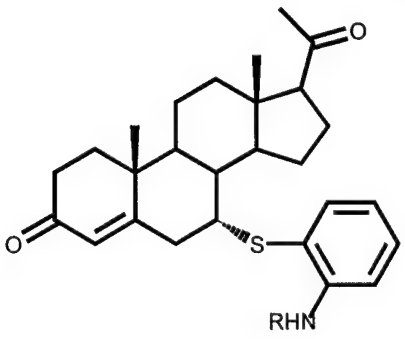


Fig. 10. Structure of progesterone and of its PgA compounds (C-7 progesterone analogs). For the structure of the R function, see Tables ...

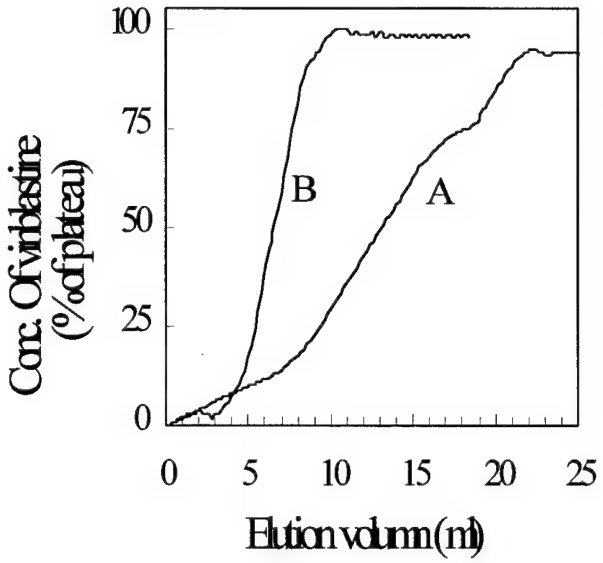


Fig. 11. Frontal chromatography of VBL on the PGP-SP

Selected Reprints from this Award

1. Leonessa, F., Kim, J.-H., Ghiorghis, A., Kulawiec, R. Hammer, C., Talebian, A. & Clarke R. 7C-7 Analogs of progesterone as potent inhibitors of the P-Glycoprotein efflux pump. *J Med Chem,* in press.

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C-7 Analogues of Progesterone as Potent Inhibitors of the P-Glycoprotein Efflux Pump

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Received March 20, 2001

The P-glycoprotein product (Pgp) of the MDR1 gene has been implicated in the multiple drug resistance phenotype expressed by many cancers. Functioning as an efflux pump, P-glycoprotein prevents the accumulation of high intracellular concentrations of substrates. We have taken a rational approach to designing inhibitors of P-glycoprotein function, selecting a natural substrate (progesterone) as our lead compound. We hypothesized that progesterone, substituted at C-7 with an aromatic moiety(s), would exhibit reduced Pgp affinity, significantly increased antiPgp activity, and reduced affinity for progesterone receptors (PGR). We synthesized 7α-[4′-(aminophenyl)thio]pregna-4-ene-3,20-dione (**2**), which comprises a C-7α thiol bridge linking an aminophenyl moiety to progesterone, from pregna-4,6-diene-3,20-dione (**1**). The subsequent addition reaction of **2** with the appropriate isocyanate produced an initial series of compounds (**3**−**6**). Compounds **3**−**5** (respectively, $-CH_2CH_2Cl$; $-CH_2CH_3$; and $-CH(CH_3)C_6H_5$) exhibit a significantly increased ability to inhibit P-glycoprotein. Potency for restoring doxorubicin accumulation in MDR1-transduced human breast cancer cells is increased up to 60-fold as compared with progesterone. Compound **5** has greater potency than verapamil and is equipotent with cyclosporin A, for inhibiting P-glycoprotein function. Furthermore, **5** does not bind to PGR, implying a potential reduction in in vivo toxicity. These data identify C-7-substituted progesterone analogues and **5**, in particular, as rationally designed antiPgp compounds worthy of further evaluation/development.

Introduction

While many cancers are initially responsive to cytotoxic chemotherapy, most acquire a resistant phenotype. This phenotype is often characterized by crossresistance to structurally unrelated drugs to which the tumor has not been exposed. The precise genes that confer this multidrug resistance phenotype are unknown, but there are several strong single-gene candidates. These include several ABC transporters including the P-glycoprotein product of the MDR1 gene (Pgp; gp170),[1] the lung resistance protein,[2] the breast cancer resistance protein,[3] and several members of the multidrug resistance associated protein family.[4,5] The precise contribution of each potential multidrug resistance mechanism is unclear. Indeed, more than one mechanism may operate, either within the same tumor cell subpopulation and/or within different subpopulations of the same tumor.

We have chosen to study Pgp-mediated multiple drug resistance. Pgp confers resistance to drugs by preventing their accumulation within the cell. Pgp's efflux capabilities appear to reflect its ability to bind substrates within the inner leaflet of the plasma membrane.[6] Subsequently, and in a potentially adenosine 5′-triphosphate (ATP)-dependent manner, substrates are expelled from the cell.[7] We have shown by meta analysis that Pgp expression is detected in ≥50% of breast cancers and that this expression is associated with prior chemotherapy, a worse than partial response to chemotherapy, and in vitro resistance to Pgp substrates.[8] These data suggest that where Pgp expression is detected, it likely contributes to multiple drug resistance in some breast cancers. Nonetheless, the likely role of Pgp in conferring drug resistance remains controversial.

The poor activity of current antiPgp agents in patients has been attributed to the presence of resistance factors in addition to Pgp, inappropriate design of clinical trials, toxicity, and/or lack of specificity of antiPgp reagents. The ability of reversing agents to alter the pharmacokinetics of the coadministered cytotoxic drugs, and an inability to achieve adequate levels of some reversing agents, also are problematic.[9] The absence of a series of nontoxic drugs, specifically designed to reverse Pgp, limits the design of clinical trials to reverse this form of multidrug resistance.

One aspect of the controversy regarding the role of Pgp comes from the relatively poor activity of those few Pgp reversing agents evaluated in clinical trials. Most attention has focused on the Pgp reversing agents verapamil, cyclosporin A, and its nonimmunosuppressant analogue PSC833 (valspodar), but the activity of other drugs also has been studied in patients. Few of these compounds were designed as Pgp inhibitors. Thus, severe side effects, often related to either the "normal" function of these agents and/or their ability to influence

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10.1021/jm010126m CCC: $22.00
Published on Web 12/14/2001

additional targets, may be induced at concentrations required to affect Pgp function. Many antiPgp drugs affect the pharmacokinetics of the substrate, significantly increasing cytotoxic drug-induced toxicity.[10] Other antiPgp agents cannot readily be delivered at doses that produce adequate serum levels. For example, the serum levels of verapamil required to produce in vitro reversal of Pgp resistance are rarely achieved in patients, despite administering sufficient doses of verapamil to induce significant cardiotoxicity.[9,11,12] Adequate serum trifluoperazine levels are not reached in patients at doses that induce dose-limiting toxicities.[9,13] Peak plasma levels of the stereoisomer of *cis*-flupenthixol (*trans*-flupenthixol) are 1000-fold less than that necessary to achieve full chemosensitization in vitro.[14,15] Several clinical studies have used patient populations where tumor Pgp expression is unknown, complicating a clear determination of its contribution to multiple drug resistance.

Previously, we have established cellular breast cancer models in which to study Pgp-mediated efflux and evaluate inhibitors of this activity. These models have been generated by inducing a constitutive expression of Pgp, following transduction with retroviral gene expression vectors.[16,17] The major advantage of these models is that unlike cells selected for resistance in vitro, Pgp expression is the only mechanism present to produce the multiple drug resistance phenotype. For example, the widely used MCF7^{ADR} cells, which were selected in vitro for resistance to doxorubicin (DOX) and recently redesignated NCI/ADR-RES,[18] exhibit increased glutathione transferase and topoisomerase II activities.[19,20] Differences in the potency of isomers of fluphenthixol identified in MCF7^{ADR} cells could not be confirmed in MDR1-transfected NIH 3T3 cells.[15]

Using our cellular models, we now describe an initial series of progesterone analogues that exhibit significantly increased antiPgp activity as compared with progesterone and verapamil and comparable to that seen with cyclosporin A. Importantly, the most potent of these analogues has lost its ability to activate progesterone receptors (PGR) and is predicted to exhibit relatively low intrinsic toxicity in vivo.

Chemistry

Conceptualization and Design. We wished to take a rational, structure−function-based approach to design inhibitors of Pgp function. Initially, we hypothesized that a natural substrate for the pump could provide an ideal candidate for rational drug design, since it is likely that Pgp evolved specifically to efflux such molecules. Evidence shows that several molecules with a steroid nucleus are Pgp substrates.[21−23] Pgp is expressed in the uterus[23,24] and the placenta,[25] suggesting a natural role for protecting secretory cells from the toxic effects of high local concentrations of steroids. Progesterone is the most potent of the steroids, including progesterone's metabolites, for reversing the effects of Pgp expression.[23,26,27] Progestins have intrinsically lower toxicity than other reversing agents and are orally active. In addition, progesterone is readily available and cheap, and the chemistry for generating several structural modifications is relatively straightforward.[28,29] Thus, we chose progesterone as our lead compound.

The major beneficial properties we wished to confer included but were not restricted to (i) improved potency for Pgp reversal, (ii) either no change or a reduction in affinity for PGR, and (iii) no agonist (mitogenic) activities. Concurrently, we wished to avoid either a substantial increase in PGR binding or a loss of Pgp reversing potency.

Unfortunately, the precise structure−function characteristics of Pgp reversing agents are unknown. This is not surprising, given the remarkable structural diversity of Pgp substrates.[30] Nonetheless, several characteristics are apparent, providing generic guidelines for the design of Pgp reversing agents. Lipophilicity appears central, with increased lipophilicity strongly associated with increased antiPgp activity.[31−37] Planar aromatic rings are commonly found in substrates, and these may contribute to lipophilicity.[37] Amphipathicity also is common, as is the presence of a basic amine, where primary amines appear most effective.[31,33,35,36,38] Size, for example, as determined by calculated molar refractivity, appears an important factor in several classes of compounds.[32,35,39] C21-aminosteroids have a structural similarity to progesterone, and in these compounds, the steroid moiety, lipophilicity, and amphipathicity are considered important attributes.[40] For compounds composed of two structures joined by a molecular spacer, the length of the spacer seems important.[15,31,34,41,42] This suggests that some part of the molecule may be oriented into a "pocket" in Pgp.[31] This pocket may have specific requirements for lipophilicity, size, and charge.

A C-7 addition to the steroid 17β-estradiol, as occurs in the antiestrogens ICI 182,780 and ICI 164,384, produces compounds with low toxicity and potentially appropriate pharmacokinetics.[43,44] Limited evidence suggests that ICI 164,384 can reverse Pgp-mediated resistance,[45] despite the apparent inability of 17β-estradiol to do so.[23] Thus, a bulky C-7 substitution on a steroid nucleus might increase antiPgp activity. C-7-substituted progesterone analogues were synthesized 20 years ago, but several exhibit antiprogestational activity.[46] Data from these studies suggest that bulky additions at C-7, when these include an aromatic ring, reduce PGR affinity by approximately 10−1000-fold.[46] Consequently, it may be possible to reduce the endogenous toxicity of progesterone by reducing/eliminating its ability to bind PGR.

On the basis of the various structure−function observations noted above, we hypothesized that progesterone, substituted at C-7 with an aromatic moiety(s), would exhibit both reduced Pgp affinity and significantly increased antiPgp activity. Thus, we designed an initial compound from which we could derive an appropriate series of progesterone analogues for evaluation. This compound, 7α-[4′-(aminophenyl)thio]pregna-4-ene-3,20-dione (**2**), has a C-7 thiol bridge linking an aminophenyl moiety to progesterone. Subsequent additions to the amine with the appropriate isocyanate generated the corresponding Pgp analogues. For our initial series of compounds, we selected isocyanates that would provide analogues with predicted differences in the size, lipophilicity, and charge of their C-7 additions.

Synthesis. Compound **1** (Scheme 1) was prepared from progesterone by a modified Turner and Ringold's

Scheme 1. Synthesis of C-7 Progesterone Analogues

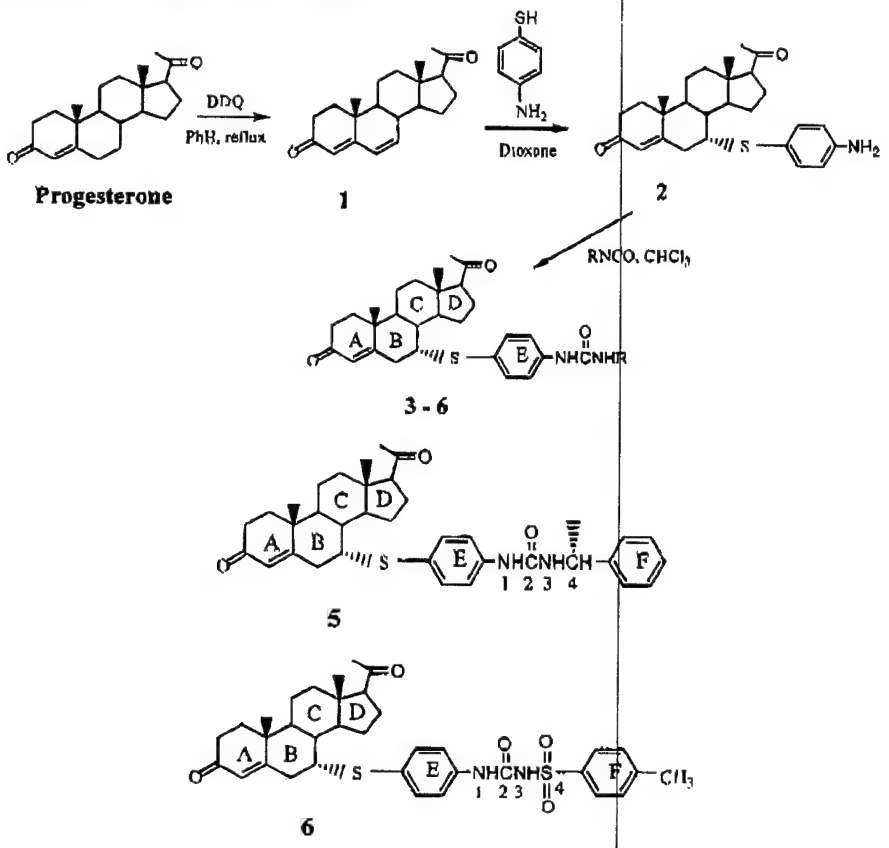

Table 1. Structure and Physical Properties of C-7 Progesterone Analogues[a]

compd	R	mp (deg)	R_f[b]
2	N/A	228–230	0.23
3	$-CH_2CH_2Cl$	137–141	0.47
4	$-CH_2CH_3$	130–135	0.36
5	$-CH(CH_3)C_6H_5$	146–149	0.46
6	$-SO_2C_6H_4CH_3$	128–132	0.29

[a] See Scheme 1 for the structures of **2**–**6**. [b] R_f in hexane–ethyl acetate (2:3).

method,[47-49] using 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) as an oxidizing agent and *p*-toluenesulfonic acid (*p*-TsOH) in refluxing benzene via Dean–Stark distillation. Purification of the crude **1** on silica gel gives 6-dehydroprogesterone (**1**) as a yellow solid (35%. R_f = 0.44, 2:3 hexanes–ethyl acetate, mp = 143–145 °C). Reaction of compound **1** with 4-aminothiophenol and NaOH pellets, in degassed dioxane as solvent for 6 days at 74 °C, provided 7α-[(4′-aminophenyl)thio]pregna-4-ene-3,20-dione (**2**) as an ivory solid. Crude crystals of **2** were precipitated from a mixture of hexanes–ethyl acetate and purified by flash column chromatography to yield 790 mg of white solid (61%, R_f = 0.23, 2:3 hexanes–ethyl acetate, mp = 228–230 °C).

The additional C-7 progesterone analogues (**3**–**6**) were obtained by reacting compound **2** with the appropriate isocyanate (Table 1). The general reaction was performed under a N_2 atmosphere for 12 h, until no **2** was detected by thin-layer chromatography (TLC), and the solvent was removed under reduced pressure. All crude analogues were purified by flash column chromatography to yield the corresponding ureas as a white solid (40–83%). The physical properties of these analogues are provided in Table 1 as mp and R_f on silica gels (2:3 hexanes–ethyl acetate).

Results and Discussion

Substrate Accumulation Studies. Because Pgp is an efflux pump, we measured the ability of our compounds to influence the intracellular accumulation of the cytotoxic drugs vinblastine (VBL) and DOX. Both drugs are widely used clinically and are efficiently effluxed by Pgp.[50] Activity was evaluated in MDR1-transduced human breast cancer cells (MDA435/LCC6^{MDR1}), using the parental cells (MDA435/LCC6) as the Pgp-negative control. Potency of the compounds was compared with that of progesterone and the established Pgp inhibitors verapamil and cyclosporin A. VBL content in Pgp-positive cells, exposed to media containing 5 nM [^{3}H] VBL, was approximately 6-fold lower than in parental Pgp-negative cells. Cellular content of DOX, in cells exposed to 4 μM DOX, was about 8-fold lower in the presence than in the absence of Pgp.

Results of VBL and DOX accumulation studies, summarized in terms of EC_{50}, are presented in Table 2. Because these data were estimated from dose response curves, representative curves are shown in Figure 1. Treatment with progesterone analogues **3**–**6** reverses the difference in VBL and DOX content between Pgp-positive and Pgp-negative cells. Analogues **3**–**5** exhibit significantly increased antiPgp potency as compared

Table 2. Potency of C-7 Progesterone Analogues, Progesterone, Verapamil, and Cyclosporin A in Reversing the Difference in VBL and DOX Accumulation between Pgp-Negative and Pgp-Positive Cells

	reversal of [^{3}H] VBL accumulation		reversal of DOX accumulation	
compd	EC_{50} μM[a] (relative potency)[b]	Pgp-specific EC_{50}[c] μM (relative potency)	EC_{50} μM (relative potency)	Pgp-specific EC_{50} μM (relative potency)
progesterone	18.7 ± 3.7[d] (1)	21.0 ± 4.2 (1)	22.3 ± 2.0 (1)	42.2 ± 7.2 (1)
3	0.8 ± 0.2 (22.5)	0.9 ± 0.2 (23.5)	0.6 ± 0.1 (40.5)	0.7 ± 0.2 (60.2)
4	1.3 ± 0.1 (14.2)	1.5 ± 0.2 (14.0)	0.7 ± 0.07 (31.3)	1.0 ± 0.06 (42.7)
5	0.8 ± 0.2 (24.5)	0.7 ± 0.2 (28.2)	0.6 ± 0.07 (37.2)	0.9 ± 0.09 (44.8)
6	34.8 ± 8.6 (0.5)	33.4 ± 5.2 (0.6)	14.7 ± 3.2 (1.5)	37.0 ± 6.5 (1.1)
verapamil	1.2 ± 0.2 (16.1)	3.1 ± 0.9 (6.8)	2.4 ± 0.3 (9.2)	4.1 ± 0.5 (10.2)
cyclosporin A	0.6 ± 0.06 (32.5)	0.6 ± 0.06 (32.5)	0.5 ± 0.1 (41.9)	0.7 ± 0.2 (60.6)

[a] EC_{50} = drug concentration that reduces the difference in drug accumulation between MDA435/LCC6 and MDA435/LCC6^{MDR1} cells by 50%; obtained by interpolation on dose response curves. Representative curves are shown in Figures 1 ([^{3}H] VBL) and 2 (DOX). [b] Values in parentheses represent the potency of each compound relative to the lead compound progesterone. [c] Pgp-specific EC_{50} = data corrected for any effect of test compound on drug accumulation in MDA435/LCC6 (Pgp-negative) cells. [d] Values represent the mean ± SE obtained from at least three independent experiments.

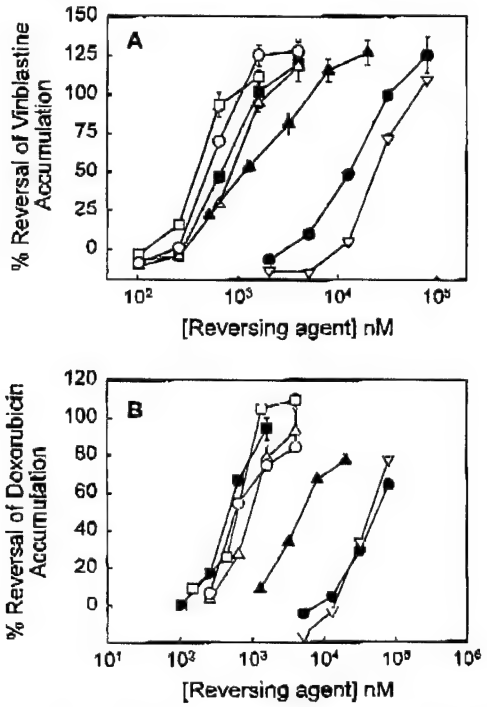

Figure 1. Ability of C-7 progesterone analogues to affect [^{3}H] VBL accumulation (A) and DOX accumulation (B) in MDA435/LCC6 and MDA435/LCC6^{MDR1} cells. Data (mean ± SE) are from one of three or more representative experiments used to obtain the ED_{50} values presented in Table 2. Progesterone = ●, cyclosporin A = ○, verapamil = ▲, **3** = ■, **4** = △, **5** = □, and **6** = ▽.

with progesterone, being 14−60-fold more potent. In marked contrast, **6** is only equipotent with progesterone. Three compounds (**3**−**5**) are significantly more potent than verapamil when Pgp-specific EC_{50}s are compared for both VBL and DOX accumulation. Compounds **3** and **5** were equipotent with cyclosporin A ($p > 0.05$ for all comparisons). Recently, we have established a chromatographic approach for assessing relative Pgp binding affinities.[51,52] Studies to measure the affinity of these analogues are in progress.

While **3** and **5** tend to be slightly more potent than **4**, the difference is not statistically significant. This suggests that addition of either a Cl (**3**) or a second aromatic ring (**5**) does not further increase activity. In marked contrast, the presence of the sulfonyl group in **6** eliminates the gain in activity conferred by the C-7 moiety. Thus, the increased activity in **5** is not simply due to the presence of an aromatic F ring (Scheme 1). Further structural modifications will allow us to test further the structure−activity relationships of C-7 progesterone analogues for Pgp reversal.

A major problem with many existing antiPgp compounds is their intrinsic toxicity. We wished to obtain an in vitro assessment of the toxicity of our compounds relative to progesterone, verapamil, and cyclosporin A. We used our breast cancer cell models because they do not express PGR and would provide a simple model for assessing PGR-independent cytotoxicity. Furthermore, any reduction in toxicity seen in the MDA435/LCC6^{MDR1} cells, as compared with the MDA435/LCC6 cells (relative resistance of Pgp-positive cells in Table 3), would suggest that the compounds were Pgp substrates, not simply Pgp inhibitors. Results are summarized in terms of IC_{50} in Table 3; representative dose response curves are shown in Figure 2.

To estimate relative activity, each drug's intrinsic cytotoxicity was expressed relative to its antiPgp activity (IC_{50}/EC_{50}; Table 3). We did not detect cytotoxicity for compounds **4** and **5**, due to their low solubility, rendering our ratios underestimates based on the highest (noncytotoxic) concentration tested. Nonetheless, **4** produces ~40% inhibition of proliferation at 20 μM. In marked contrast, 20 μM **5** does not inhibit proliferation significantly in either untreated cells or MDA435/LCC6 and MDA435/LCC6^{MDR1} cells.

When adjusted for cytotoxicity, cyclosporin A and progesterone exhibit approximately equivalent relative activities. The low estimates for cyclosporin A reflect its substantial toxicity. Compound **6** is the least active compound, and **5** is the most active despite the overestimation of its cellular toxicity. While VBL and DOX may have different recognition sites in Pgp,[53] **5** shows broadly comparable activity against both drugs, as does cyclosporin A.

Having established that the C-7 addition significantly increased antiPgp activity, we wished to evaluate the PGR activity of our best compound. Overall, **3** and **5** have antiPgp activity comparable to cyclosporin A. Because **3** exhibits significant cellular toxicity, we chose to evaluate the relative affinity of **5** for binding to PGR. We compared the ability of **5**, progesterone, and unlabeled ORG2058, a synthetic progestin, to compete with [^{3}H] ORG2058 for binding to PGR. The data in Figure

Table 3. Growth Inhibitory Activity of C-7 Progesterone Analogue, Progesterone, Verapamil, and Cyclosporin A on MDA435/LCC6 (Pgp-Positive) and MDA435/LCC6^{MDR1} (Pgp-Negative) Human Breast Cancer Cells

	IC_{50} μM			IC_{50}/EC_{50}[c]	
compd	MDA435/LCC6 (relative cytotoxicity)[a]	MDA435/LCC6^{MDR1} (relative cytotoxicity)	relative resistance of Pgp-positive cells[b]	VBL activity	DOX activity
progesterone	27.4 ± 7.9[d] (1.0)	36.4 ± 8.3 (1.0)	1.4 ± 0.09	1.3	0.6
3	3.2 ± 0.09 (8.5)	7.3 ± 2.5 (5.0)	2.2 ± 0.7	3.6	3.2
4	>20.0 (ND)[e]	>20.0 (ND)	ND	>13.3	>20.0
5	>20.0 (ND)	>20.0 (ND)	ND	>28.6	>22.2
6	22.1 ± 1.6 (1.2)	38.2 ± 0.2 (1.0)	1.7 ± 0.1	0.7	0.6
verapamil	65.8 ± 0.04 (0.4)	63.4 ± 1.2 (0.6)	1.0 ± 0.02	21.2	16.0
cyclosporin A	1.0 ± 0.5 (26.9)	1.1 ± 0.2 (33.6)	1.3 ± 0.4	1.7	1.4

[a] Relative cytotoxicity = ability of compounds to inhibit cell growth relative to progesterone. [b] Relative resistance of Pgp-positive = ratio of toxicity in resistant and control cells. Relative resistance > 1 suggests that the compound is at least partly effluxed by Pgp. [c] This ratio relates the effect of each drug on either VBL or DOX accumulation to its intrinsic cellular toxicity; higher ratios suggest a greater degree of safety. [d] Values represent the mean ± SE obtained from at least three independent experiments. [e] ND = no data; an IC50 was not reached at the highest concentration tested (limited by solubility). Some values (>) are underestimates based on nontoxic concentrations.

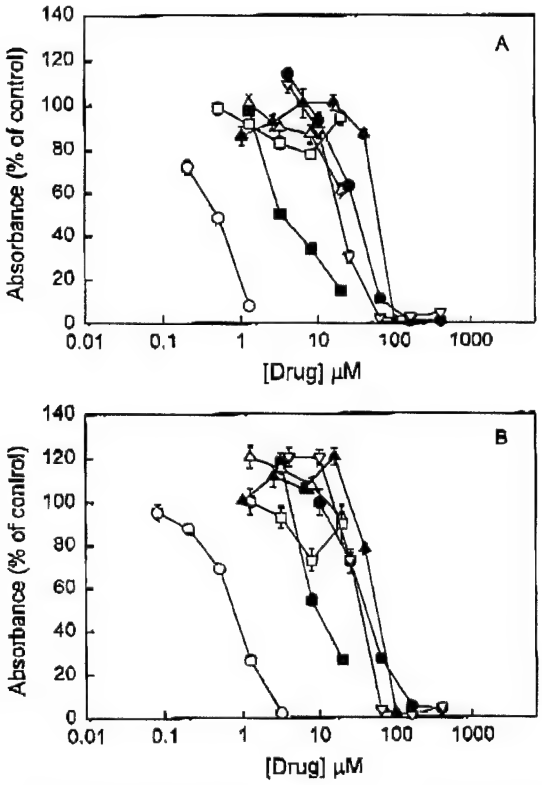

Figure 2. Cytotoxicity of progesterone analogues in MDA435/LCC6 (A) and MDA435/LCC6^{MDR1} cells (B). Data (mean ± SE) are from one of three or more representative experiments used to obtain the IC_{50} values presented in Table 3. Progesterone = ●, cyclosporin A = ○, verapamil = ▲, **3** = ■, **4** = △, **5** = □, and **6** = ▽.

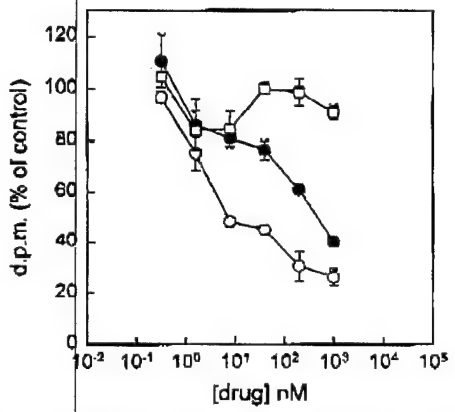

Figure 3. Competitive binding of progesterone, ORG2058, and **5** to PGR. [^{3}H] ORG2058 was used as the radiolabeled ligand. Progesterone = ●, ORG2058 = ○, and **5** = □.

3 show that **5**, at concentrations up to its EC_{50}, does not significantly compete with ORG2058. This represents a reduction of >100-fold in its PGR affinity as compared with ORG2058. Thus, **5** has antiPgp activity comparable to cyclosporin A, exhibits potentially low intrinsic cellular toxicity, and does not bind to its predicted cellular target (PGR) at its EC_{50} for inhibition of Pgp activity.

Conclusions

While we cannot draw definitive structure–activity conclusions, some potentially useful preliminary observations can guide future studies. The molecules are clearly amphipathic, with lipophilicity greatest around the "E" and/or "F" rings and the polarity greatest around C-17–C-21. These observations suggest that effective substrates may concurrently interact with both hydrophilic and hydrophobic regions. However, it is not clear whether these are both in Pgp as previously suggested[31] or whether they represent pockets at the plasma membrane/Pgp interface. The possibility that drugs are removed from within the plasma membrane[6] may favor the model that invokes a plasma membrane component to the binding interaction.

Compounds **3**–**5** are significantly more potent than progesterone at specifically increasing Pgp substrate accumulation. These observations are consistent with our initial hypothesis that aromatic C-7 substitutions of progesterone will increase activity and with the known contribution of aromatic moieties in other modulating agents.[37,42] Compound **6** also contains a C-7 aromatic addition but is essentially equipotent with progesterone. Perhaps the simplest explanation is that this compound is the least lipophilic of the analogues, since lipophilicity appears to be a major factor in the activity of other Pgp substrates.[31–35]

The significant increase in potency observed with compounds **3**–**5** supports our initial structure–function-based hypothesis, based on previous published observations. The activity of our existing compounds already compares well with that of cyclosporin A. C-7 progest-

erone analogues have the potential to provide more potent, selective, and safe inhibitors of Pgp function than others that have currently completed clinical trials. We believe that the observations reported here, combined with the lack of receptor binding activity, identify **5** as the next logical lead compound for further development and provide valuable clues for the further optimization of this structure. We are currently synthesizing a larger series of compounds to further optimize the MDR1 reversing potency and effectively define the structure–activity relationships of these compounds.

Our ability to increase the potency of progesterone up to 60-fold (**3**; Pgp-specific EC_{50} for DOX accumulation) supports the use of relatively limited structure–function data in the design of effective antiPgp compounds. Furthermore, by including structure–activity information on the binding characteristics of the lead's natural intracellular target (PGR) in our conceptualization, we reduced affinity of **5** for a target that could produce toxicity in normal cells. We are now poised to evaluate our compounds in vivo, to pursue further modifications that may increase antiPgp activity, and to explore the structure–activity relationship for C-7 progesterone analogues in detail. Overall, the data in this study identify C-7-substituted progesterone analogues and **5**, in particular, as rationally designed antiPgp compounds worthy of further evaluation/development.

Experimental Section

Chemistry. General Procedures. All reactions were carried out under an atmosphere of nitrogen using standard Schlenk techniques.[54] Benzene and chloroform were distilled from CaH_2, stored over 3D molecular sieves, and deaerated by purging with nitrogen immediately before use. TLC was performed using Merck glass plates precoated with F_{254} silica gel 60; compounds were visualized by UV and/or with *p*-anisaldehyde stain solution. Flash chromatography was performed using EM Science silica gel 60, following the procedure of Still,[55] with the solvent mixtures indicated. Melting points were measured on a Thomas-Hoover capillary melting point apparatus and are uncorrected (Table 1). The broad melting points for compounds **3**–**6** suggest the presence of minor impurities. All reagents were purchased from commercial suppliers and used as received unless indicated otherwise. Dioxane was purchased from Aldrich in Sure-Seal bottles.

Nuclear magnetic resonance (NMR) spectra were measured on Nicolet NT 270 and Varian Mercury 300 MHz instruments at the Georgetown NMR Facility. Chemical shifts are reported in units of parts per million relative to Me_4Si. All spectra are recorded in $CDCl_3$. Significant ^{1}H NMR data are tabulated in the following order: multiplicity (s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet), coupling constants in Hertz, and number of protons. ^{13}C NMR spectra were recorded at frequencies of 67.9 and 75.6 MHz. Infrared (IR) spectra were measured on a MIDAC Corp. or a Mattson Galaxy 2020 Series FTIR, as neat films; absorption bands are reported in cm^{-1}. Low-resolution mass spectra were measured on a Fisons Instruments MD 800 quadrupole mass spectrometer, with 70 EV electron ionization and a GC 8000 Series gas chromatograph inlet, and using a J & W Scientific DB-5MS column (15 m length, 0.25 mm internal diameter, 0.25 μm film thickness). Mass spectra data are given as mass-to-charge ratio, with the relative peak height following in parentheses. All new compounds were characterized by ^{1}H NMR, IR, and ^{13}C NMR spectroscopies. Fast atom bombardment mass spectra (FABMS) were recorded at the University of Maryland College Park of Mass Spectrometry Facility. Literature references are given for all known compounds, except for those that are commercially available; all known compounds were identified by ^{1}H NMR spectroscopy. Elemental analysis was performed by Atlantic Microlab (Norcross, GA).

Pregna-4,6-diene-3,20-dione (1). Compound **1** was prepared by the method of Tuner and Ringold.[47,48] Thus, *p*-TsOH monohydrate (11.0 g, 63.9 mmol) was dehydrated in freshly distilled benzene (320 mL) by azeotropic refluxing using a Dean–Stark trap. After 1 h, the solution was cooled for 0.5 h, and progesterone (5.0 g, 15.9 mmol) and DDQ (4.6 g, 20.3 mmol) were added. The olive mixture was refluxed for 3 h and then filtered through a pad of Celite. The filtrate was washed with saturated NaCl (5 × 20 mL) followed by 1% NaOH solution until it gave clear solution and dried over anhydrous $MgSO_4$. Solvent was removed under reduced pressure, and the filtrate was purified by chromatography; 1.69 g of product (35%. R_f = 0.44, 2:3 hexanes–ethyl acetate); yellow solid (mp = 143–145 °C). ^{1}H NMR: δ 6.12 (s, 1H), 5.69 (s, 1H), 2.84–1.12 (complex, 12H), 2.17 (s, 3H), 2.14 (s, 3H), 1.12 (s, 3H), 1.10 (s, 1H), 1.00 (s, 1H), 0.72 (s, 3H). IR: 3855, 3745, 3678, 2953, 1700, 1663, 1457, 1361, 1223, 875, 754.

7α-[4′-(Aminophenyl)thio]pregna-4-ene-3,20-dione (2). We obtained **2** using the method of Brueggemeier et al.[56] Briefly, **1** (1.65 g, 5.28 mmol), 4-aminothiophenol (1.32 g, 10.56 mmol), and NaOH (pellet, 116 mg, 2.9 mmol) were placed in a Schlenk tube, which was purged with a constant flow of N_2(g). Deoxygenated anhydrous dioxane (25 mL) was added and heated at 74 °C for 6 days. The mixture was concentrated under reduced pressure and purified by chromatography; 790 mg white solid (61%. R_f = 0.23, 2:3 hexanes–ethyl acetate); mp = 228–230 °C). ^{1}H NMR: δ 7.26–7.21 (q, J = 8.5 Hz, 2H), 6.64–6.61 (q, J = 8.5 Hz, 2H), 5.73 (s, 1H), 3.77 (s, 2H), 3.24 (s, 1H), 2.14 (s, 3H), 2.63–1.10 (complex, 11H), 1.19 (s, 3H), 0.69 (s, 3H). IR: 3420, 3360, 3250, 2930, 1700. ^{13}C NMR: δ 209.3, 199.0, 167.6, 147.1, 136.6, 127.3, 121.2, 115.7, 63.4, 17.7, 13.1.

General Procedure for the Preparation of Progesterone Analogues. A suspension of **2** in degassed chloroform was treated with the appropriate isocyanates under N_2. The mixture was stirred for 12 h and then chromatographed directly on silica gel to afford the corresponding ureas as oil. The resulting oil was stirred in anhydrous ether until white powder came out.

7α-[4′-(*N*-Chloroethylaminoacylaminophenyl)thio]pregna-4-ene-3,20-dione (3). Reaction of **2** (0.10 g, 0.23 mmol) with 2-chloroethylisocyanate (38 μL, 0.46 mmol) in $CHCl_3$ (3.0 mL) for 12 h gave 50 mg of product (40%, mp = 137–141 °C, R_f = 0.47, 2:3 hexanes–ethyl acetate). ^{1}H NMR: δ 7.34–7.25 (m, 4H), 5.69 (s, 1H), 5.18 (s, 1H), 3.68–3.62 (m, 4H), 3.38 (s, 1H), 2.64–0.84 (complex, 18H), 2.14 (s, 3H), 1.19 (s, 3H), 0.69 (s, 3H). IR: 3312, 2964, 1700, 1630, 1587, 1517, 1488, 1449, 1394, 1238, 1013, 831, 734. ^{13}C NMR: δ 231.5, 210.3, 196.2, 193.9, 181.9, 156.5, 149.3, 146.4, 141.4, 132.9, 125.1, 119.5, 118.5, 103.2, 94.2, 75.9, 75.8, 71.9, 69.3, 49.0, 35.8, 24.2, 14.4. MS: m/e = 543 (24, M^+ + 1), 507 (10), 313 (27), 230 (23), 185 (50), 149 (69), 125 (57), 119 (23), 107 (38), 105 (48), 91 (50), 81 (50), 57 (73), 55 (100). HRMS: calcd for $C_{30}H_{39}N_2O_3SCl$ $[M + H]^+$, 543.24481; found, 543.24248. Anal. Calcd for ($C_{30}H_{40}O_3N_2SCl$): C, 66.22; H, 7.41; N, 8.82; S, 6.52. Found: C, 66.38; H, 7.27; N, 8.78; S, 6.28.

7α-[4′-(*N*-Ethylaminoacylaminophenyl)thio]pregna-4-ene-3,20-dione (4). Reaction of **2** (0.10 g, 0.23 mmol) with ethylisocyanate (37 μL, 0.46 mmol) in $CHCl_3$ (3.0 mL) for 12 h gave 78 mg of product (67%, mp = 130–135 °C, R_f = 0.36, 2:3 hexanes–ethyl acetate). ^{1}H NMR: δ 7.36–7.25 (m, 4H), 6.38 (s, 1H), 5.69 (s, 1H), 4.18–4.03 (m, 2H), 3.38–3.26 (m, 2H), 2.67–0.68 (complex, 17H), 2.14 (s, 3H), 2.05 (s, 2H), 1.20 (s, 3H), 0.69 (s, 3H). IR: 3855, 3745, 3678, 3373, 2953, 2359, 1700, 1663, 1539, 1457, 1223. ^{13}C NMR: δ 228.5, 222.5, 193.9, 171.5, 141.5, 135.0, 128.3, 127.0, 123.4, 118.5, 108.7, 96.2, 84.5, 69.3, 67.7, 66.0, 62.6, 52.2, 48.4, 46.3, 43.9, 39.8, 34.1, 22.9, 21.2, 13.4. MS: m/e = 509 (62, M^+ + 1), 438 (8), 313 (32), 196 (47), 125 (100), 117 (57), 97 (52), 95 (85), 79 (68), 71 (59). HRMS: calcd for $C_{30}H_{40}N_2O_3S$ $[M + H]^+$, 509.28378; found, 509.28372. Anal. Calcd for ($C_{30}H_{41}O_3N_2S$): C, 70.69; H, 8.11; N, 5.49; S, 6.29. Found: C, 70.46; H, 8.06; N, 5.52; S, 6.20.

7α-[4′-(*N*-α-(+)-Methylbenzylaminoacylaminophenyl)-thio]pregna-4-ene-3,20-dione (5). Reaction of **2** (0.10 g, 0.23 mmol) with (*R*)-(+)-α-methylbenzylisocyanate (66 μL, 0.46 mmol) in $CHCl_3$ (3.0 mL) for 12 h gave 56 mg of product (46%, mp = 146−149 °C, R_f = 0.46, 2:3 hexanes−ethyl acetate). ^{1}H NMR: δ 7.32−7.25 (m, 5H), 5.79−5.77 (m, 1H), 5.70−5.68 (s, 1H), 4.97−4.92 (m, 1H), 4.13−4.06 (m, 1H), 3.28 (s, 1H), 2.64−1.49 (complex, 7H), 2.14 (s, 3H), 1.45 (d, J = 9.3 Hz, 3H), 1.19 (s, 3H), 0.68 (s, 3H). IR: 3353, 3273, 2949, 2854, 2362, 2340, 1700, 1653, 1595, 1539, 1457, 1460, 1376, 1343, 1159, 1089, 916. ^{13}C NMR: δ 209.4, 199.0, 167.6, 147.0, 136.6, 127.2, 121.2, 115.8, 63.4, 17.7, 13.1. MS: *m/e* = 585 (11, M^+ + 1), 135 (12), 125 (20), 105 (100), 103 (22), 91 (29), 77 (22), 55 (26). HRMS: calcd for $C_{36}H_{41}N_2O_3S$ [M + H]$^+$, 585.31506; found, 585.31501. Anal. Calcd for ($C_{36}H_{45}O_3N_2S$): C, 73.81; H, 7.74; N, 4.78; S, 5.47. Found: C, 73.76; H, 7.79; N, 4.81; S, 5.39.

7α-[4′-(*N*-*p*-Toluenesulfonylaminoacylaminophenyl)-thio]pregna-4-ene-3,20-dione (6). Reaction of **2** (0.10 g, 0.23 mmol) with *p*-toluenesulfonylisocyanate (59 μL, 0.46 mmol) in $CHCl_3$ (3.0 mL) for 12 h gave 120 mg of product (83%, mp = 128−132 °C, R_f = 0.29, 2:3 hexanes−ethyl acetate). ^{1}H NMR: δ 8.38 (s, 1H), 7.88 (d, J = 8.4 Hz, 2H), 7.80 (d, J = 8.3 Hz, 2H), 7.37−7.25 (m, 4H), 5.70 (s, 1H), 3.36 (s, 1H), 2.67−1.13 (complex, 20H), 2.41 (s, 3H), 2.15 (s, 3H), 1.55 (m, 2H), 1.20 (s, 3H), 0.69 (s, 3H). IR: 3855, 3745, 2359, 1700, 1539, 1457, 1160, 1086, 668. ^{13}C NMR: δ 198.6, 148.6, 141.4, 136.6, 134.6, 129.9, 129.7, 129.6, 127.7, 127.2, 126.4, 120.5, 118.5, 92.4, 76.1, 69.3, 63.3, 52.1, 51.1, 47.0, 46.3, 39.8, 39.4, 38.5, 38.1, 35.4, 34.0, 31.6, 23.7, 22.9, 21.8, 21.1, 17.9, 13.4. MS: *m/e* = 635 (29, M^+ + 1), 313 (39), 155 (33), 135 (36), 125 (65), 119 (64), 91 (100), 85 (92), 77 (47), 59 (50), 47 (45). HRMS: calcd for $C_{35}H_{42}N_2O_5S_2$ [M + H]$^+$, 635.26135; found, 635.26130. Anal. Calcd for ($C_{35}H_{43}O_5N_2S_2$): C, 66.11; H, 6.82; N, 4.41; S, 10.09. Found: C, 66.05; H, 6.79; N, 4.45; S, 10.02.

Pharmacology. Cell Lines. For the studies of antiPgp activity, we used cells transduced with a retroviral vector directing the constitutive expression of the Pgp gene (MDA435/LCC6^{MDR1}) and their parental, Pgp-negative, MDA435/LCC6 human breast cancer cells.[17] Both MDA435/LCC6 and MDA435/LCC6^{MDR1} cells are estrogen receptor and PGR negative and grow as monolayer cultures in vitro and as rapidly proliferating solid tumors and malignant ascites in vivo in nude mice.[17] We used MCF-7 human breast cancer cells[57] to measure binding to PGR. These cells were routinely grown in vitro in Improved Minimal Essential Media (Biofluids) containing 5% fetal bovine serum in a 5% CO_2:95% air atmosphere.

Substrate Accumulation Assays. Pgp reversing activity of all test agents was evaluated by measuring the ability of the agents to affect accumulation of DOX and VBL in MDA435/LCC6^{MDR1} (resistant) and MDA435/LCC6 (control) cells. Cells were plated at 2.5 × 10^5 cells/well into 24 well culture dishes, in routine growth media, and incubated at 37 °C. Forty-eight hours after they were plated, cells were treated by replacing growth media with media containing the test compounds at five different concentrations and either DOX (1 μM) or [^{3}H] VBL (5 nM). All treatments were carried out in triplicate. After 3 h of incubation, treatments were stopped by washing each well with 0.5 mL of ice-cold NaCl (0.15 M). Cells from reference wells in each plate were counted to enable accumulation to be corrected for cell number.

For the DOX accumulation assays, DOX was extracted from cell monolayers by adding 1.5 mL of 20% trichloroacetic acid to each well and incubating overnight at 4 °C in the dark. DOX concentrations in the extracts were evaluated fluorometrically. Thus, extracts were transferred into 13mm × 100 mm borosilicate glass tubes, placed in the 10 × 10 rack of a Hitachi A3000 Autosampler and connected to a Hitachi F-4500 fluorescence spectrophotometer. Fluorescence was read at 500 nm excitation and 580 nm emission wavelengths. Concentrations of DOX were obtained by interpolation on a DOX standard curve and normalized on the extraction volume and number of cells per well. For the VBL accumulation assays, at the end of treatment, wells were rinsed with phosphate-buffered saline (0.5 mL/well) and left to dry at room temperature. Cell monolayers were removed by trypsinization and diluted with 10 mL of scintillation fluid (Ultima Gold XR, Packard Bioscience, Meriden, CT). Drug accumulation was radiometrically assessed by scintillation spectrometry.

Results of substrate accumulation assays were plotted as cellular concentration of substrate against the concentration of the respective test compound. Pgp reversing potency was expressed as the EC_{50}, defined as the concentration of a test drug that reduced the difference in substrate accumulation between Pgp-negative and Pgp-positive cells by 50%. Progesterone and the standard Pgp reversing agents verapamil and cyclosporin A were used as positive controls and as a reference to establish relative potency.

Cytotoxicity. Twenty-four hours after they were plated in 96 well plates, MDA435/LCC6 and MDA435/LCC6^{MDR1} cells were exposed to growth media containing different concentrations of the test agents for 5 days. Cell cultures were then fixed/stained by incubation in a 0.5% (w/v) crystal violet solution in 25% methanol (v/v). After plates had dried, the dye was extracted in 0.1 M sodium citrate in 25% methanol (v/v) and absorbance was read at 540 nm using a microplate spectrophotometer. Absorbance directly correlates with cell number in this assay.[50] Cell survival curves were obtained by plotting absorbance values (as percent of untreated controls) against drug concentration. The toxicity of each drug was expressed as an IC_{50}, defined as the concentration inhibiting cell density by 50% at the end of the treatment period. To estimate the extent of resistance conferred by Pgp, the ratio of each drug's IC_{50} in MDA435/LCC6^{MDR1} and MDA435/LCC6 cells (relative resistance of Pgp-positive cells) was used for those drugs that produced a detectable IC_{50} value.

Radioligand Binding Studies. These were performed as previously described, using a whole cell competitive binding assay.[57,58] Briefly, MCF-7 cells were grown in 24 well dishes and incubated at 37 °C with 100 nM hydrocortisone for 30 min, before determining PGR binding, to eliminate residual binding to glucocorticoid receptors. Subsequently, cells were incubated for 60 min at 37 °C with 5 nM [^{3}H] ORG2058 (specific activity 50.6 Ci/mmol) in the absence or presence of increasing concentrations of unlabeled competitor (0.5 nM−1 μM; progesterone, ORG2058, 5). Radioactivity was extracted into ethanol and measured in a liquid scintillation spectrometer.

Data Analysis. DOX accumulation and cytotoxicity dose response data were processed and graphed using SigmaPlot 4.0 (SPSS Science, Chicago, IL). EC_{50} (DOX accumulation assays) and IC_{50} values (cytotoxicity assays) were calculated by interpolation on the respective dose response curves. The EC_{50} and IC_{50} values reported in Tables 2 and 3 represent the mean and standard error (SE) obtained from at least three independent experiments. Descriptive statistics were obtained using SigmaStat 2.0 (SPSS Science).

Acknowledgment. This work was supported in part by the Department of Defense, United States Army Medical Research and Materiel Command, Award RP950649, and by a grant from the Susan G. Komen Breast Cancer Foundation, Award PDF 2000 186.

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JM010126M

MSC: jm010126m MTY: a

0 **C-7 Analogues of Progesterone as Potent Inhibitors of the P-Glycoprotein Efflux Pump**

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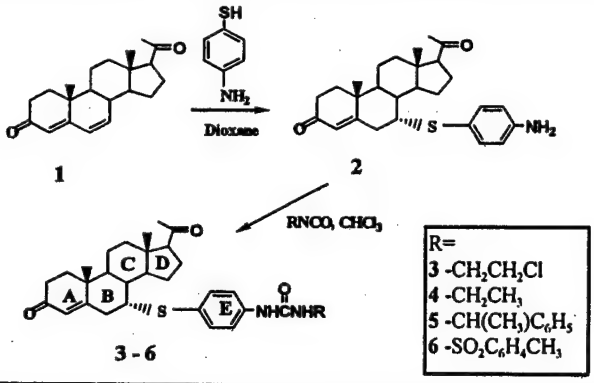

Synopsis Depth: 144q

The Enantioselective Binding of Mefloquine Enantiomers to P-Glycoprotein Determined Using an Immobilized P-Glycoprotein Liquid Chromatographic Stationary Phase

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Received May 17, 2001; accepted May 24, 2001

KEY WORDS: P-glycoprotein; enantiomers of mefloquine; dissociation constant of drugs; immobilized receptor; frontal affinity chromatography.

INTRODUCTION

Mefloquine (MQ), α-2-piperidinyl-2,8-bis(trifluoromethyl)-4-quinolinemethanol (Fig. 1), is an antimalarial agent widely used to treat chloroquine-resistant malaria (1). The agent is administered as a racemic mixture of erythro-isomers, (+)-[11R,2′S]-MQ [(+)-MQ] and (−)-[11S,2′R]-MQ [(−)-MQ]. In humans, there is an enantioselective distribution of MQ with higher plasma and brain concentrations of (−)-MQ (2,3).

MQ has also been shown to inhibit the activity of the drug efflux transporter P-glycoprotein (Pgp) (4–7). Shao *et al.* demonstrated that MQ increased the intracellular accumulation of the Pgp substrate daunomycin in the P388/ADR leukemia cell line (4). In addition, when MQ was used concomitantly with the Pgp-substrate vinblastine (VBL), the two agents interacted with each other synergistically in a noncompetitive manner.

However, MQ has also been shown to increase the intracellular accumulation of VBL (6,7). In rat brain capillary endothelial GPNT cells, the inhibition of VBL transport was enantioselective with (+)-MQ displaying up to an eightfold greater effect than (−)-MQ (6,7). In Caco-2 human colon carcinoma cells, both (−)-MQ and (+)-MQ significantly increased cellular accumulation of VBL, but the effect was not enantioselective (6,7).

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ABBREVIATIONS: Pgp: P-glycoprotein; VBL: vinblastine; CsA: cyclosporin A; MQ: mefloquine.

The objective of this study was to examine the molecular basis of the observed effect of MQ on VBL transport in light of the observed combined effects of MQ and VBL on daunomycin intracellular accumulation. The experimental approach examined the effect of (+)-MQ and (−)-MQ on the Pgp binding affinities of [^{3}H]-VBL and [^{3}H]-cyclosporine A ([^{3}H]-CsA). The studies were carried out using an immobilized Pgp liquid chromatographic stationary phase (Pgp-SP) (8). The Pgp-SP was constructed using membranes derived from a human cell line transduced with a retroviral vector directing the expression of MDR1 (8). This phase has been used to determine ligand-Pgp binding affinities and to investigate ligand-ligand binding interactions on Pgp (8,9).

The experimental approach was competitive binding studies using (+)-MQ and (−)-MQ as the displacers and [^{3}H]-VBL or [^{3}H]-CsA as the ligand. The results demonstrate that the addition of either (+)-MQ or (−)-MQ completely suppressed the binding of [^{3}H]-VBL to Pgp, in a manner indicative of an anticooperative allosteric interaction. There was no observed enantioselectivity in this process. However, when [^{3}H]-CsA was the marker ligand, (+)-MQ competitively displaced this marker, whereas (−)-MQ had no effect. This indicates that, under the experimental conditions, MQ enantioselectively binds [(+)-MQ > (−)-MQ] at a site at which [^{3}H]CsA binds to Pgp. This is the first observation of enantioselective binding to human-derived Pgp.

MATERIALS AND METHODS

Materials

[^{3}H]VBL and [^{3}H]CsA were purchased from Amersham Life Science Products (Boston, MA). VBL, CsA, CHAPS, glycerol, and benzamidine were from Sigma Chemical Co. (St. Louis, MO). (+)-MQ and (−)-MQ were kindly provided by Hoffmann La Roche (Basel, Switzerland). Scintillation liquid (Flo-Scint V) was purchased from Packard Instruments (Meriden, CT). The chromatographic backbone (immobilized artificial membrane PC stationary phase, IAM.PC) was obtained from Regis Chemical Co. (Morton Grove, IL). The HR5/2 glass column was purchased from Amersham Pharmacia Biotech (Uppsala, Sweden).

Preparation of the Pgp-SP

The Pgp-SP was prepared as previously described (8,9). In brief, the Pgp-positive membranes were obtained from the MDA435/LCC6^{MDR1} human breast cancer cell line (10). The cultured cells (8×10^7 cells) were harvested in 10 ml of buffer (Tris-HCl [50 mM, pH 7.4], 50 mM NaCl, 2 μM leupeptin, 2 μM phenylmethanesulfonyl fluoride, and 4 μM pepstatin). The suspension of cells was homogenized for 2 × 30 s, centrifuged at 1,000 × g for 10 min, and the supernatant was collected and centrifuged at 150,000 × g for 30 min.

The resulting pellets were added to 6 ml of Tris-HCl (50 mM, pH 7.4) containing 500 mM NaCl, 15 mM CHAPS, 2 mM DTT, and 10% glycerol. After 3 h at 4°C, the solution was mixed with 100 mg of dried IAM PC stationary phase, stirred for 1 h at room temperature, and then dialyzed against Tris-HCl (10 mM, pH 7.4) containing 150 mM NaCl, 1 mM EDTA, and 1 mM benzamidine for 36 h at 4°C (1.5 l·12 h).

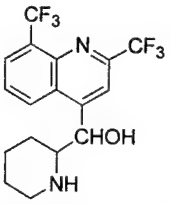

Fig. 1. The structure of mefloquine.

The resulting Pgp-SP was packed into a 0.5 (id) × 0.8 cm HR5/2 glass column. The resulting column was equilibrated with Tris-HCl (50 mM, pH 7.4) at room temperature for 3 h.

Frontal Chromatographic Studies Using Online Flow Scintillation Detection

The chromatographic system has been described (8,9). Detection of the marker ligands, [^{3}H]-VBL and [^{3}H]-CsA, was accomplished using an online flow scintillation monitor (Radiometric FLO-ONE® Beta 500 TR instrument, Packard Instruments). All chromatographic experiments were conducted at room temperature using a flow rate of 0.5 ml/min. The running buffers used in these studies were composed of Tris-HCl buffer (50 mM, pH 7.4) with and without the concomitant addition of 3 mM ATP.

The marker ligand, either 1.0 nM [^{3}H]-VBL or 2.0 nM [^{3}H]-CsA, was applied to the Pgp-SP in sample volumes of 25–50 ml. Before the initiation of a new series of studies, column performance was assessed by applying 1.0 nM [^{3}H]-VBL to the Pgp-SP. The appearance of specific frontal curves with a reproducible retention volume (~30 ml) indicated that there had been no degradation of column performance.

The solutions containing the marker ligands were supplemented with a range of concentrations of either cold VBL, CsA , (+)-MQ, or (−)-MQ. Elution profiles were obtained showing front and plateau regions. The observed elution volume data were used for calculation of ligand dissociation constants. The K_d value of (+)-mefloquine was calculated by nonlinear regression using Prism (GraphPad Software) and a one-site binding (hyperbola), Equation 1 (9,11).

$$Y = Bmax \bullet X/(K_d + X) \quad (1)$$

where X is the concentration of marker ligand (in the present experiment, it is VBL or CsA); Y is equal to [ligand] (V − Vmin), where Vmin is the elution volume of VBL or CsA under conditions where specific interactions are completely suppressed; and V is the retention volume of VBL or CsA at different concentrations of MQ (0.2–1.0 μM).

RESULTS AND DISCUSSION

The Effect of (+)-MQ and (−)-MQ on VBL Binding

When [^{3}H]-VBL was added to the running buffer and chromatographed on the Pgp-SP, frontal and plateau regions were obtained, Fig. 2A, trace 1, and Fig. 2B, trace 1. The relatively flat or shallow increase in the curve during the initial elution volume (0 to 30 ml) followed by a sharp increase in concentration of the marker (breakthrough) followed, in turn, by a plateau is indicative of a specific binding interaction between [^{3}H]-VBL and the immobilized Pgp.

When 0.2 μM (+)-MQ or 0.2 μM (−)-MQ were added to the running buffer, the specific frontal regions were lost from the [^{3}H]-VBL chromatograms (Fig. 2A, trace 2, and Fig. 2B, trace 2, respectively). These results indicate that the specific binding interaction between [^{3}H]-VBL and the immobilized Pgp had been lost. If MQ had competitively displaced [^{3}H]-VBL, the chromatographic traces would have mirrored the frontal curves seen in trace 1 (Figs. 2A and 2B), but the breakthrough volumes would have been reduced. The change in the shapes of the curves is consistent with an anticooperative allosteric interaction. In this process, MQ binds to a contiguous or separate site on the Pgp molecule producing secondary effects at the VBL binding site that reduce the ability of VBL to bind to that site.

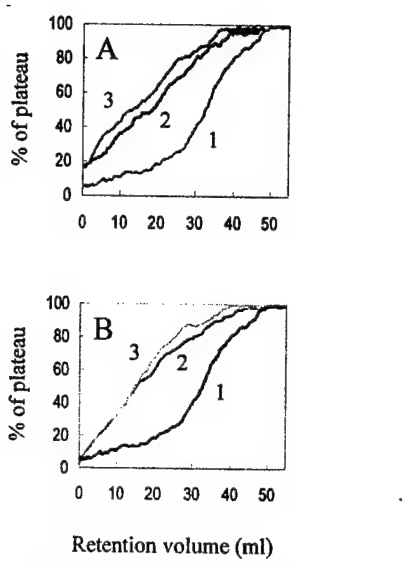

Fig. 2. The effect of MQ enantiomers on the frontal affinity chromatography of 1.0 nM [^{3}H]-VBL on the Pgp-SP where: **(A)** Trace 1: [^{3}H]-VBL alone; Trace 2: 200 nM (+)-MQ added to the running buffer, Trace 3: 200 nM (+)-MQ plus 3 mM ATP added to the running buffer. **(B)** Trace 1: [^{3}H]-VBL alone; Trace 2: 200 nM (−)-MQ added to the running buffer, Trace 3: 200 nM (−)-MQ plus 3 mM ATP added to the running buffer. The running buffer was 50 mM Tris-HCl (50 mM, pH 7.4).

Anticooperative allosteric effects on the binding of VBL to immobilized Pgp have been observed after the addition of 3 mM ATP to the running buffer (9). In the present study, the addition of 3 mM ATP to a running buffer containing either 0.2 μM (+)-MQ or 0.2 μM (−)-MQ produced no significant change in the observed VBL chromatograms (Fig. 2A, trace 3, and Fig. 2B, trace 3, respectively). These results support the observation that the effect produced by the addition of MQ to the running buffer was allosteric in nature and not competitive.

Shao *et al.* (4) demonstrated that MQ and VBL cooperate to reduce daunomycin transport and that VBL did not significantly affect the K_i of MQ relative to daunomycin transport. The authors concluded that MQ and VBL act at physically different sites on the Pgp molecule. The hypothesis developed in the current study that MQ affects VBL through an anticooperative allosteric interaction is consistent with these conclusions. In addition, previous studies with the Pgp-SP (9) have demonstrated that VBL competitively displaced doxorubicin, a compound closely related to daunomycin. Thus, the two-site synergism of MQ and VBL relative to

daunomycin transport can be explained by a competitive displacement produced by VBL at the site at which daunomycin binds to Pgp and an anticooperative allosteric interaction produced by MQ binding to a different site.

At the MQ concentrations used in this study (0.2–1.0 μM), both (−)-MQ and (+)-MQ had equivalent effects on VBL binding and no enantioselectivity was observed. The lack of enantioselectivity is consistent with the results from the studies in Caco-2 cells but not with the eightfold greater effect of (+)-MQ observed in rat brain capillary endothelial GPNT cells (6.7). The source of the difference in MQ enantioselectivity between the Caco-2 and GPNT cell lines is not clear and may simply reflect a species-dependent variation in Pgp (12).

The Effect of (+)-MQ and (−)-MQ on CsA Binding

Previous studies with the Pgp-SP have demonstrated that the presence of 3 mM ATP in the running buffer is required to produce specific binding interactions between CsA and the immobilized Pgp (9). This effect appears to be produced through a cooperative allosteric interaction. Therefore, in the present investigation, when [^{3}H]-CsA was the marker ligand, all of the chromatographic experiments were conducted with 3 mM ATP in the running buffer.

In these experiments, increasing concentrations of MQ from 0.2 to 1.0 μM were added to the running buffer. This concentration range reflects maximum steady-state plasma concentrations of (−)-MQ (~4.0 μM) and (+)-MQ (~0.7 μM) (3). In addition, MQ concentrations of >10 μM saturated the Pgp-SP.

The addition of increasing concentrations of (+)-MQ to the running buffer produced corresponding reductions in [^{3}H]-CsA retention volumes. Although the retention volumes decreased, the specific frontal chromatograms were retained (Fig. 3), signifying the existence of a competitive binding interaction between (+)-MQ and [^{3}H]-CsA. Using Eq. 1, the K_d value for (+)-MQ was calculated to be 471 ± 146 nM, which is consistent with the K_i value of 410 nM determined for the effect of MQ on daunomycin accumulation in P388/MDR cells (4).

Addition of increasing concentrations of (−)-MQ did not effect the retention volume of [^{3}H]-CsA, nor was there a change in the specific frontal chromatograms (data not shown). This indicates that (−)-MQ was not able to competitively displace [^{3}H]-CsA. Therefore, there is an enantioselective difference in the affinities of the MQ enantiomers, (+)-MQ > (−)-MQ, at a site at that [^{3}H]-CsA binds to Pgp. To our knowledge, this is the first report of enantioselective binding to Pgp.

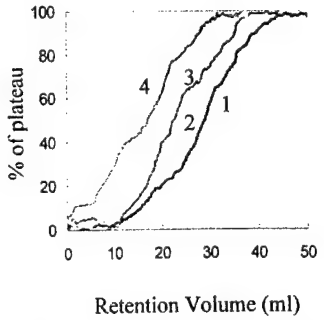

Fig. 3. The effect of (+)-MQ on the frontal affinity chromatography of 2.0 nM [^{3}H]-CsA on the Pgp-SP where: 1) represents the elution profile of [^{3}H]-CsA without (+)-MQ in the running buffer; 2) after addition of 0.5 μM (+)-MQ; 3) after addition of 0.8 μM (−)-MQ; 4) after addition of 1.0 μM (−)-MQ. The running buffer was Tris-HCl (50 mM, pH 7.4) containing 3 mM ATP.

CONCLUSIONS

Enantioselectivity at a site at which CsA binds to Pgp presents two avenues for further investigation of this site. Because the physicochemical properties of enantiomers are equivalent, (−)-MQ can be used to determine nonspecific interactions with Pgp allowing for a clearer picture of the specific binding interactions between (+)-MQ and Pgp. In addition, enantioselective binding at this site also provides a three-dimensional probe that can be used in pharmacophore modeling.

The data from this study also demonstrate that MQ and VBL bind to separate but allosterically interconnected sites on the Pgp-SP. This model is consistent with previous data obtained with the Pgp-SP demonstrating that CsA and VBL have separate, but closely linked binding sites on the Pgp molecule (9). Because the affect of MQ on VBL binding was anticooperative and not enantioselective, (+)-MQ, (−)-MQ, or the racemic mixture could be used to produce the synergistic effects on Pgp transport reported by Shao *et al.* (4). However, this does not hold for CsA Because (+)-MQ had a greater effect on CsA binding to Pgp than (−)-MQ. Thus, the enantioselectivity of the MQ interactions with Pgp present a number of possible clinical options for paired inhibitors. For example, if VBL is the target, then one might use a combination of (−)-MQ and CsA and, conversely, if CsA is the target, one might use (+)-MQ and VBL. The results suggest that competitive binding experiments on the Pgp-SP may be a rapid method for the identification of synergistic pairs for specific clinical targets.

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0026-895X/01/5901-62–68$3.00
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 59:62–68, 2001

Vol. 59, No. 1
248/875317
Printed in U.S.A.

Competitive and Allosteric Interactions in Ligand Binding to P-glycoprotein as Observed on an Immobilized P-glycoprotein Liquid Chromatographic Stationary Phase

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Received June 8, 2000; accepted September 29, 2000

This paper is available online at http://molpharm.aspetjournals.org

ABSTRACT

A liquid chromatographic stationary phase containing immobilized P-glycoprotein (Pgp) was synthesized using cell membranes obtained from Pgp-expressing cells. The resulting Pgp-stationary phase was used in frontal and zonal chromatographic studies to investigate the binding of vinblastine (VBL), doxorubicin (DOX), verapamil (VER), and cyclosporin A (CsA) to the immobilized Pgp. The compounds were added individually to the chromatographic system with or without ATP in the running buffer. Using this approach, dissociation constants were calculated for VBL (23.5 ± 7.8 nM), DOX (15.0 ± 3.2 μM), VER (54.2 ± 4.7 μM), and CsA [97.9 ± 19.4 nM (without ATP) and 62.5 ± 4.6 nM (with ATP)]. The compounds were also added in pairs using standard competitive chromatography procedures. The results of the study demonstrate that competitive interactions occurred between VBL and DOX, cooperative allosteric interactions occurred between VBL and CsA and ATP and CsA, and anticooperative allosteric interactions occurred between ATP and VBL and VER. The chromatographic studies indicate that the immobilized Pgp was modified by ligand and cofactor binding and that the stationary phase can be used to study drug-drug binding interactions on the Pgp molecule.

P-glycoprotein (Pgp) is a member of the ATP binding cassette (ABC) superfamily of transport proteins (Loe et al., 1996; Doyle et al., 1998). It is a 170-kDa cell membrane protein with two ATP binding sites and ATPase activity (Rosenberg et al., 1997). Pgp acts as an efflux drug transporter whose substrates include anthracycline antibiotics and Vinca alkaloids (Cordon-Cardo et al., 1989; Clarke et al., 1993; Clarke and Leonessa, 1994), steroids (Barnes et al., 1996), verapamil (VER) (Yusa and Tsuro, 1989), peptides (Foxwell et al., 1989), and quinolines (Kusuhara et al., 1997). Pgp is expressed in normal tissues and appears to be a major contributor to the blood-brain barrier (Cordon-Cardo et al., 1989; Tsuji et al., 1992). Expression also has been detected in breast cancer where it is associated with a poor clinical response (Trock et al., 1997).

Pgp's broad substrate specificity has not been definitively explained. Several indirect and direct models for Pgp activity have been proposed (Shapiro and Ling, 1994). The most popular model is the "membrane vacuum cleaner" mechanism in which Pgp binds its substrate from the inner leaflet of the plasma membrane and releases it into the extracellular fluid (Gottesman and Pastan, 1993). In a related mechanism, Pgp activity has been described as a "flippase" that transports its substrates from the inner to the outer leaflet of the plasma membrane (Raviv et al., 1990; Higgins and Gottesman, 1992).

The number of binding sites on the Pgp molecule has not been determined. There is evidence for the existence of multiple binding sites as some substrates bind to Pgp in a mutually noncompetitive manner (Raviv et al., 1990; Ferry et al., 1992, 1995). Other data suggesting multiple binding sites include synergistic activity on ATPase activation (Garrigos et al., 1997), substrate discriminating effect of specific Pgp mutations (Devine et al., 1992), and differential effect of chemosensitizers on the photoaffinity labeling at two different locations on the Pgp molecule (Dey et al., 1997).

One experimental approach to determine Pgp selectivity and transport mechanism has been the isolation of the transporter followed by purification using a combination of anion exchange and affinity chromatography (Shapiro and Ling, 1994; Sharom, 1995). The isolated protein was then reconstituted into proteoliposomes either by the detergent dilution method (Shapiro and Ling, 1994) or by detergent dialysis followed by Sephadex-G50 chromatography (Sharom, 1995). In the proteoliposomes prepared by either method, >90% of Pgp was reconstituted with an inside-out orientation, i.e., ATP-binding and cytoplasmic domains exposed to the ex-

This research was supported by National Institutes of Health Grant 2R42M56591-02 (I.W.W.).

ABBREVIATIONS: Pgp, P-glycoprotein; VBL, vinblastine; DOX, doxorubicin; VER, verapamil; CsA, cyclosporin A; IAM, Immobilized Artificial Membrane; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonate; BCA, bicinchoninic acid.

travesicular medium (Sharom, 1995). The reconstituted Pgp could be used to study and characterize both drug-stimulated ATPase activity and ATP-dependent transport. Using this approach, the effect of verapamil and daunorubicin on [^{3}H]vinblastine ([^{3}H]VBL) accumulation in the proteoliposomes, a measure of transport, could be measured (Sharom, 1995). The effect of verapamil on the ATPase kinetics (K_m and V_{max}) also could be determined (Shapiro and Ling, 1994).

Another approach to the determination of the effect of compounds on Pgp transport used the transepithelial flux of digoxin across Caco-2 cells (Wandel et al., 1999). This method was used to determine the IC_{50} for digoxin transport for 14 compounds. An in vivo method for Pgp transport in tumors and the blood-brain barrier also has been reported (Hendrikse et al., 1999). This approach used [^{11}C]verapamil and [^{11}C]daunorubicin as the transport substrates and positron emission tomography as the detection method.

The binding of compounds to Pgp has been investigated by measuring the displacement of [^{3}H]vinblastine and [^{3}H]verapamil from human intestinal Caco-2 cells overexpressed with Pgp (Doppenschmitt et al., 1999). The assays were performed in 96-well plates, and the method was designed to be adapted to high-throughput screens. Using this method, K_m and IC_{50} values for nine compounds were determined.

An alternative experimental approach to the determination of binding affinities is affinity chromatography. We have previously reported the synthesis of a liquid chromatographic stationary phase containing immobilized Pgp and its use in the determination of Pgp binding affinities (Zhang et al., 2000). The present work expands the characterization of the Pgp-stationary phase and uses frontal and zonal chromatographic techniques to investigate the binding of vinblastine, doxorubicin, verapamil, and cyclosporin A (CsA) to the immobilized Pgp. The compounds were added individually to the chromatographic system with or without ATP in the running buffer. The compounds were also added in pairs using standard competitive chromatography procedures. The results of the study demonstrate that both competitive and allosteric interactions occurred during the chromatographic studies and that the binding affinities of immobilized Pgp are altered by the presence or absence of ATP.

Experimental Procedures

Materials. Immobilized Artificial Membrane (IAM) particles were obtained from Regis Chemical Co. (Morton Grove, IL). A glass column (HR5/5) was purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). [^{3}H]Vinblastine and [^{3}H]cyclosporin A were purchased from Amersham Life Science Products (Boston, MA). [^{3}H]Verapamil was from NEN Life Science Products, Inc. (Boston, MA). Vinblastine, verapamil, doxorubicin, cyclosporin, CHAPS, glycerol, benzamidine, and bovine serum albumin were from Sigma Chemical Co. (St. Louis, MO). GF/C glass microfiber filters were from Whatman (Ann Arbor, MI). Scintillation liquid (Flo-Scint V) was purchased from Packard Instruments (Meriden, CT).

Preparation of Membranes. As previously described, the Pgp-positive MDA435/LCC6^{MDR1} cell line was obtained by transduction of Pgp-negative-expressing MDA435/LCC6 human breast cancer cells with a retroviral vector carrying MDR1 cDNA (Pgp) (Leonessa et al., 1996). Approximately 8×10^7 cells were harvested in 10 ml of buffer A (50 mM Tris-HCl, pH 7.4, 50 mM NaCl, 2 μM leupeptin, 2 μM phenylmethanesulfonyl fluoride, and 4 μM pepstatin). The suspension of cells was homogenized twice for 30 s (with a cooling period in between) with a Brinkmann (Westbury, NY) Polytron homogenizer. The homogenized cells were centrifuged first at 1,000g for 10 min, the pellets were discarded, and the supernatant was collected and centrifuged at 150,000g for 30 min. The membrane pellets were collected.

Immobilization of Pgp on IAM Particles. The membrane pellets were resuspended in 6 ml of solubilization solution (50 mM Tris-HCl, pH 7.4, 500 mM NaCl, 15 mM CHAPS, 2 mM dithiothreitol, 10% glycerol) for 3 h at 4°C. This was mixed with 100 mg of dried IAM particles and stirred for 1 h at room temperature. The suspension of Pgp-IAM was then dialyzed against dialysis buffer (150 mM NaCl, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM benzamidine) for 36 h at 4°C (1.5 liters for every 12 h).

Preparation of the Liquid Chromatographic Column. The IAM particles with immobilized Pgp were packed into a HR5/5 glass column (0.5 × 0.8 cm) after centrifugation three times at 350g for 3 min at 4°C. Then the column was equilibrated with buffer B (50 mM Tris-HCl, pH 7.4) at room temperature for 3 h.

Frontal Chromatographic Studies. The chromatographic system has been previously described (Zhang et al., 2000) and was primarily based upon the Pgp-IAM column connected on-line to a flow scintillation monitor (Radiometric FLO-ONE Beta 500 TR instrument; Packard Instruments). All chromatographic experiments were conducted at room temperature using a flow rate of 0.5 ml/min.

The marker ligand, either [^{3}H]VBL (1.0 nM), [^{3}H]VER (0.3 nM), or [^{3}H]CsA (2.0 nM) were applied to the Pgp-IAM column in sample volumes of 25 to 50 ml. The solutions containing the marker ligands were supplemented with a range of concentrations of either cold VBL, VER, doxorubicin, or CsA. Elution profiles were obtained showing front and plateau regions as illustrated for [^{3}H]VER (Fig. 1). The observed elution volume data were used for calculation of ligand dissociation constants. The K_d values of VER and CsA were calculated by nonlinear regression using Prism (GraphPad Software, San Diego, CA) and a one-site binding (hyperbola) equation (1) (Klotz, 1983)

$$Y = B_{max} \cdot X/(K_d + X) \quad (1)$$

in which X is the concentration of VER or CsA; Y is equal to [verapamil] $(V - V_{min})$ or [CsA]$(V - V_{min})$, where V_{min} is the elution volume of VER or CsA under conditions where specific interactions

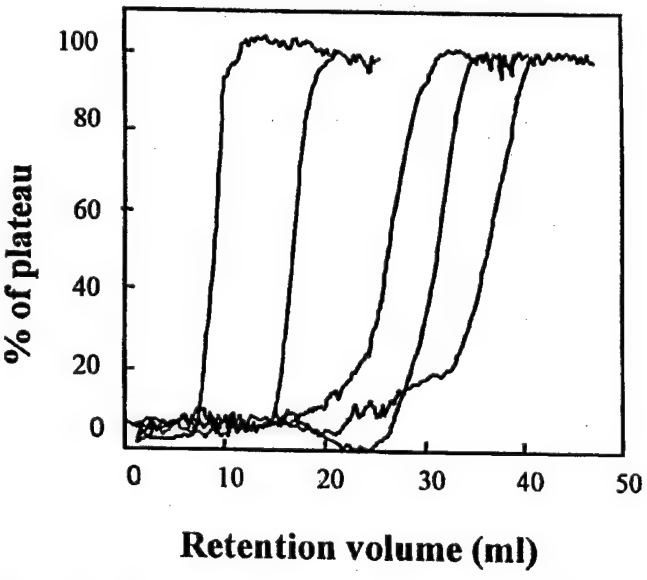

Fig. 1. Frontal analysis of interactions of Pgp with verapamil on an immobilized Pgp-IAM column (0.5 × 0.8 cm). The elution profiles of 1.0 nM [^{3}H]verapamil in solution with 10, 40, 60, 200, and 400 μM nonradioactive verapamil are shown (from right to left). Running buffer was 50 mM Tris-HCl, pH 7.4, at a flow rate of 0.5 ml/min.

are completely suppressed and V is the retention volume of VER or CsA at different concentrations (0.3–400 μM for VER and 2.5–100 nM for CsA).

Two series of runs were made to determine the K_d value for VBL and the K_d values for doxorubicin and CsA. One series was performed with cold VBL (3–100 nM) to displace [^{3}H]VBL, and the other was performed with cold doxorubicin (5–70 μM) or CsA (10–250 nM) with [^{3}H]VBL as the displaced ligand. The K_d value of VBL and the K_d values of doxorubicin and CsA were calculated using eqs. 2 and 3 (Winzor, 1985; Brekkan et al., 1996; Zhang et al., 1998).

$$(V_{max} - V)^{-1} = (1 + [VBL]K_{VBL}) \cdot (V_{min}[P]K_{VBL})^{-1} + (1 + [VBL]K_{VBL})^2 \cdot (V_{min}[P]K_{VBL}K_i)^{-1} \cdot [I]^{-1} \quad (2)$$

$$(V - V_{min}) - 1 = (V_{min}[P]K_{VBL})^{-1} + (V_{min}[P])^{-1}[VBL] \quad (3)$$

where I represents doxorubicin or CsA; $[P]$ represents the concentration of active receptor in the volume; V_{min} represents the elution volume of VBL under conditions where the specific interaction is completely suppressed; V_{max} is the elution volume obtained with 1.0 nM [^{3}H]VBL.

Control Experiments. Membranes from the Pgp-negative parental cell line MDA435/LCC6 (Leonessa et al., 1996) were prepared and immobilized on an IAM support as described above. Using the procedure described above, the Pgp-negative-IAM support was packed into a glass column (0.5 × 0.8 cm), and a second glass column (0.5 × 0.8 cm) was packed with untreated IAM support. The three columns, IAM support (negative control), Pgp-negative-IAM (positive control), and Pgp-IAM (experimental), were separately connected on-line to a flow scintillation monitor and used in zonal chromatographic experiments. In these studies, a mobile phase composed of Tris-HCl (50 mM, pH 7.4) was constantly pumped through the column at a flow rate of 0.5 ml/min. A single 100-μl injection of the marker ligand [^{3}H]VER (23.5 nM) was injected onto the column, and the radioactive signal (cpm) was recorded every 6 s. The chromatographic data was summed up in 0.5-min intervals and smoothed using the Microsoft Excel program with a 5-point moving average.

Membrane Binding Assays. The binding assays were accomplished using a previously described method (Ferry et al., 1995). Briefly, 50 μl of [^{3}H]VBL [3–100 nM with 2% ethanol (v/v)] was incubated with Pgp-containing or Pgp-negative membranes (150 μg in 50 μl) or bare IAM particles and 50 μl of cold VBL (12 μM) for 2 h at room temperature. Bound and free drug were separated by rapid filtration through Whatman GF/C filters that had been presoaked with 0.1% bovine serum albumin in Tris-HCl (50 mM, pH 7.4). The filters were then washed with 2 portions of 5 ml of ice-cold 20 mM Tris-HCl, 20 mM $MgCl_2$ buffer. The filters were dried, and retained radioactivity was quantitated by liquid scintillation counting. Specific binding was defined as the difference between total binding and nonspecific binding.

Protein Assay. The amount of membrane and the immobilized membrane were determined by bicinchoninic acid (BCA) protein assay. The sample was diluted with NaOH (0.1 M). A protein standard (0.3–37.5 μg in 50 μl) was prepared with albumin standard (Pierce, Rockford, IL). The measurement procedure followed the instruction in the Pierce BCA protein assay kit in which 20 ml of reagent A was mixed with 0.4 ml of reagent B. Aliquots (50 μl) of standards and samples were added in triplicate to a 96-well plate and 200 μl of BCA reagent (A + B) were added to each well. The standards and samples were incubated at room temperature for 3 h, and the resulting absorbance at $\lambda = 570$ nm was determined using a spectrophotometer. The amount of protein was calculated by using the Microsoft Excel program.

Results

Chromatographic Studies with Vinblastine and Doxorubicin. The dissociation constants (K_d) of VBL and doxorubicin were determined on the Pgp-IAM stationary phase using displacement chromatography with [^{3}H]VBL as the marker ligand (Table 1). The calculated K_d of VBL was 23.5 ± 7.8 nM, consistent with the previously reported values of 37.0 ± 10 nM (Ferry et al., 1995) and 36 ± 5 nM (Korzekwa et al., 1998). The K_d value of 15.0 ± 3.2 μM determined for doxorubicin was also consistent with the reported value of 31.0 ± 7.3 μM (Ferry et al., 1995).

The chromatographic results also were consistent with the results obtained from binding assays using the same membranes used in the construction of the Pgp-IAM stationary phase. In these studies, membrane extracts were prepared from the Pgp-expressing cell line MDA435/LCC6^{MDR1} and the Pgp-negative cell line MDA435/LCC6 (Hendrikse et al., 1999). VBL binding to the two membrane extracts and the IAM support was determined using a previously described rapid filtration method (Ferry et al., 1995). No specific binding was observed with the Pgp-negative cell membranes or the IAM particles, while a K_d value of 54.5 ± 40.8 nM was determined using the membranes from the Pgp-expressing cell line. The calculated affinity was consistent with the previously published value, 37 ± 10 nM, obtained using the same experimental approach (Ferry et al., 1995).

Chromatographic Studies with Verapamil and Vinblastine. When VER was used as the displacer of the [^{3}H]VBL marker ligand, the calculated K_d value for VER was 54.2 ± 4.6 μM. This value was significantly higher than the previously reported values of 0.45 ± 0.05 μM (Ferry et al., 1995) and 0.6 ± 0.18 μM (Ferry et al., 1992). When the experimental conditions were reversed and [^{3}H]VER was the marker ligand and VBL the displacer, no displacement of [^{3}H]VER was observed when 50 and 100 nM concentrations of VBL were added to the mobile phase (Table 2).

The specificity of the chromatographic interactions of VER with the immobilized Pgp were investigated through the independent immobilization of membrane extracts from the Pgp-expressing cell line and the Pgp-negative cell line on the IAM support. Zonal chromatographic studies were conducted with columns containing either the Pgp-IAM, Pgp-negative-IAM, or IAM support. When a 100-μl sample of [^{3}H]VER was injected onto the columns containing either the Pgp-negative-IAM support or the IAM support alone, the retention volumes on both columns were less than 4 ml (Fig. 2, curves

TABLE 1

The K_d values calculated using frontal affinity chromatography on the immobilized Pgp-IAM stationary phase

Drugs	K_d[a]	K_d
Vinblastine	23.5 ± 7.8 nM	37.0 ± 10 nM[b]
		36.0 ± 5 nM[c]
Verapamil	54.2 ± 4.6 μM	0.45 ± 0.05 μM[b]
Doxorubicin	15.0 ± 3.2 μM[d]	31 ± 7.3 μM[b]
Cyclosporin A	62.5 ± 5.6 nM[e]	18 ± 3.6 nM[b]
	97.9 ± 19.4 nM[d]	

[a] These values were measured in the present work by frontal affinity chromatography with immobilized Pgp-IAM.
[b] These values are from Ferry et al. (1995).
[c] This value is from Callaghan et al. (1997).
[d] These values were obtained by displacing [^{3}H]vinblastine (see *Experimental Procedures*).
[e] This value was measured when 3 mM ATP was in the running buffer.

1 and 2). The volumes of these columns (as well as the Pgp-IAM column) are 0.5 ml, thus a retention of 4 ml indicates that it takes 8 column volumes to elute the [^{3}H]VER, indicating that an interaction occurred between the solute and both of the stationary phases. On the column containing the Pgp-IAM support, the retention volume of [^{3}H]VER was >20 ml (Fig. 2, curve 3).

Chromatographic retention on biopolymer containing stationary phases is a combination of nonspecific and specific interactions. The former interactions are due to the physicochemical properties of the solute and stationary phase, i.e., electrostatic and hydrophobic interactions, while the latter (specific) interactions are due to interactions between the solute and a specific binding site(s) on the biopolymer. The 5-fold increase in retention volume between the Pgp-IAM and both the Pgp-negative-IAM and IAM support alone indicates that specific binding interactions occur between [^{3}H]VER and the immobilized membrane extracts obtained from the Pgp-expressing cells.

Chromatographic Studies with Cyclosporin A and Vinblastine. When CsA was used as the displacer of the [^{3}H]VBL marker ligand, the calculated K_d value for CsA was 97.9 ± 19.4 nM, compared with the previously reported value of 18.0 ± 3.6 nM (Ferry et al., 1995) (Table 1). When [^{3}H]CsA was used as the marker ligand and migrated alone through the Pgp-IAM, the retention volume was 7.8 ml (Table 2), and no specific retention was observed (Fig. 3A). The addition of 50 nM VBL to the running buffer increased the retention volume of [^{3}H]CsA to 15.7 ml (Table 2) and produced the expected frontal chromatogram (Fig. 3B). When the VBL concentration was increased to 100 nM, the observed retention of the frontal chromatogram increased to 18.8 ml (Fig. 3D; Table 2).

Effect of ATP on the Chromatographic Properties of the Pgp-IAM. The addition of 3 mM ATP to the running buffer resulted in changes in the retention volumes of CsA, VBL, and VER. The concentration of ATP was selected based upon the previously reported studies of the secondary and tertiary structures of reconstituted Pgp (Sonveaux et al., 1996).

In the case of CsA, the addition of ATP increased the retention volume from 7.8 to 17.5 ml (Table 2). In addition to the change in elution volume, the observed chromatogram changed from a frontal curve indicative of nonspecific retention (Fig. 3A) to a frontal chromatogram characteristic of specific retention due to binding interactions between the CsA and the immobilized Pgp-IAM (Fig. 3C). With 3 mM ATP in the running buffer, [^{3}H]CsA was displaced from Pgp by the addition of unlabeled CsA. The results from the CsA displacement studies were used to calculate a K_d value of 62.5 nM for CsA binding to the immobilized Pgp.

When VBL was the marker ligand, the addition of 3 mM ATP decreased the retention volume from 32.1 to 8.4 ml (Table 2). The presence of ATP in the running buffer also changed the observed chromatograms from a frontal curve demonstrating specific retention (Fig. 4A) to a nonspecific curve (Fig. 4B). A similar effect was observed for VER as the addition of 3 mM ATP to the running buffer reduced the elution volume from 34.2 to 5.9 ml (Table 2) with a resulting loss in specific retention, as demonstrated by the shape of the frontal curve (data not shown).

Discussion

Quantitative affinity chromatography is an extensively studied and documented approach for the measurement of ligand-protein interactions (cf. Jaulmes and Vidal-Madjar, 1989). This technique uses both frontal and zonal chromatography to perform equilibrium, thermodynamic, and kinetic studies. In addition, displacement chromatographic techniques can be used to observe binding interactions between two or more ligands binding at the same or separate sites. In this manner, competitive and allosteric (cooperative or anticooperative) interactions can be readily identified.

In this study, both zonal and frontal chromatography were used to evaluate Pgp-ligand and ligand-ligand binding interactions. Using zonal chromatography, a comparison of the chromatographic retention of verapamil, a known Pgp substrate, on the native chromatographic support and the Pgp-positive and Pgp-negative supports (Fig. 2) demonstrated that, for Pgp substrates, the observed chromatographic retentions were a function of specific interactions between the substrate and the immobilized Pgp.

The relationship between chromatographic retention on the Pgp-IAM stationary phase and Pgp binding affinity was also illustrated by comparison of substrate affinities calculated using frontal chromatography on the Pgp-IAM column and the results from classical filtration binding assays (Table 1). The initial studies in this series were conducted using [^{3}H]VBL as the marker ligand and Tris buffer (50 mM, pH 7.4) as the running buffer. Under these conditions, CsA displaced [^{3}H]VBL, producing a calculated K_d value of 97.9 nM (Table 1), which is consistent with results from filtration binding assays (Ferry et al., 1992, 1995).

The displacement of [^{3}H]VBL by CsA indicated that CsA specifically and competitively binds to immobilized Pgp, but frontal chromatography with [^{3}H]CsA alone in the running buffer produced a low retention volume, 7.8 ml (Table 2), and no detectable specific retention (Fig. 3A). This indicates that under the experimental conditions, [^{3}H]CsA did not specifically bind to immobilized Pgp. However, the addition of 50 nM VBL to the running buffer produced a classical frontal

TABLE 2

Retention volumes of [^{3}H]vinblastine and [^{3}H]cyclosporin A were obtained when 1) no ATP was present in the running buffer, 2) 3 mM ATP was added in the running buffer, 3) 50 nM cold vinblastine was supplemented in the sample (no ATP in the buffer), and 4) 100 nM cold vinblastine was in the sample (no ATP in the buffer)

Drugs	Retention Volume (ml) at			
	No ATP	3 mM ATP	50 nM Vinblastine (No ATP)	100 nM Vinblastine (No ATP)
[^{3}H]Vinblastine	32.1	8.4	11.0	9.5
[^{3}H]Verapamil	34.2	5.9	34.1	34.0
[^{3}H]Cyclosporin A	7.8	17.5	15.7 (15.4)[a]	18.8

[a] 15.7 ml was measured at the condition of no ATP present in the running buffer, and 15.4 ml was obtained when 3 mM ATP was in the running buffer.

chromatogram for [^{3}H]CsA (Fig. 3B) and increased the retention volume to 15.7 ml (Table 2). When the VBL concentration was increased to 100 nM, the retention volume also increased to 18.8 ml (Table 2; Fig. 3D).

The results from the studies with [^{3}H]VBL and [^{3}H]CsA as the marker ligands indicate that the addition of VBL to the running buffer produced a cooperative allosteric interaction in the binding process between [^{3}H]CsA and the immobilized Pgp. This suggests that the binding of VBL to the immobilized Pgp alters the protein in such a manner that the site at which CsA binds is formed or made accessible to the ligand.

The data also indicated that once the VBL-induced change had occurred CsA bound to Pgp and displaced VBL through competitive and/or anticooperative allosteric interactions. The addition of CsA to the running buffer did not change the shape of the [^{3}H]VBL frontal chromatograms, demonstrating that the displacement was competitive in nature. One explanation of these results is that the VBL-induced CsA binding site is contiguous with or part of the VBL site. Thus, CsA binding to the induced site does not directly compete with VBL for the same site but inhibits VBL binding through steric interactions. Korzekwa et al. (1998) have proposed a similar model for enzymatic inhibition and activation of cytochrome P450 isoforms. In this model, the simultaneous but independent binding of two different substrates in the active site of the enzyme results in steric interactions that produce the displacement (inhibition) or reorientation (activation) of one of the substrates.

In these studies, the addition of increasing concentrations of VER to the running buffer reduced the retention volumes of [^{3}H]VBL without changing the shapes of the frontal chromatograms. This indicates that VER competitively displaced VBL from its binding to Pgp, although the calculated K_d value was significantly higher than previously reported values (Table 1). However, VBL was unable to displace [^{3}H]VER from the immobilized Pgp. These results suggest that VER binds to two or more distinct sites on the Pgp molecule including the site at which VBL binds. Furthermore, the site common to VBL and VER is not the primary, high-affinity VER binding site. Thus, the K_d value calculated from the frontal chromatographic studies (Table 1) appears to be the sum of VER binding affinities. It could not be determined from the experimental conditions used in this study whether the VER and VBL sites are allosterically linked. Further studies will be required to select specific markers for the high- and low-affinity VER binding sites.

The existence of multiple binding sites on the Pgp molecule has been previously proposed. Using classical filtration binding assays, Ferry et al. (1992) obtained evidence of nonoverlapping binding sites for Vinca alkaloids and dihydropyridine substrates and for Vinca alkaloids and doxorubicin. Also, distinct sites for steroids and Vinca alkaloids (Garrigos et al., 1997), steroids and VER (Orlowski et al., 1996), VER and dihydropyridines (Pascaud et al., 1998), and between differ-

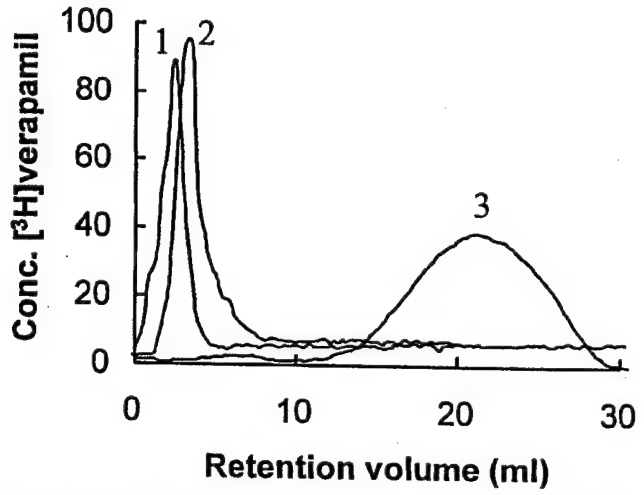

Fig. 2. Zonal affinity chromatographic profiles of 100 μl of 23.5 nM [^{3}H]verapamil at a flow rate of 0.5 ml/min with 50 mM Tris-HCl, pH 7.4, buffer. 1, from Pgp-negative-IAM column; 2, from IAM particles column; and 3, from Pgp-IAM column.

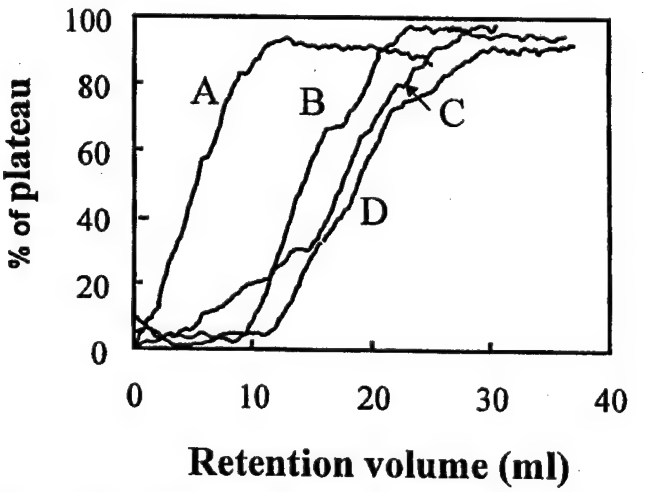

Fig. 3. Frontal affinity analysis of 1.0 nM [^{3}H]cyclosporin A. A, [^{3}H]cyclosporin A was in the sample alone; B, 50 nM cold vinblastine was supplemented in the sample; C, 3 mM ATP was in the sample and running buffer; D, 100 nM cold vinblastine was added in the sample. The running buffer was 50 mM Tris-HCl, pH 7.4.

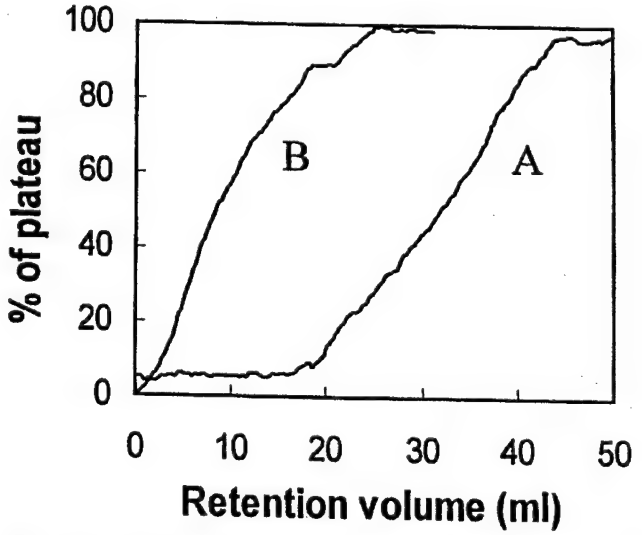

Fig. 4. Frontal affinity chromatographic analysis of 1 nM [^{3}H]vinblastine with Pgp-IAM on a column of 0.5 × 0.8 cm at a flow rate of 0.5 ml/min. A, 1.0 nM [^{3}H]vinblastine only; B, 1.0 nM [^{3}H]vinblastine supplemented with 3 mM ATP. The running buffer for both A and B was 50 mM Tris-HCl, pH 7.4, with 1.6% ethanol.

ent steroids (Orlowski et al., 1996) were supported by the results of studies using an ATPase activation endpoint. Moreover, separate binding sites have been suggested for VER and anthracyclines (Spoelstra et al., 1994; Litman et al., 1997), VER and colchicine (Korzekwa et al., 1998), and cyclosporins and dihydropyridines (Tamai and Safa, 1991).

Pgp contains two ATP binding sites (Rosenberg et al., 1997). A previous study has investigated the effect of ATP binding on the secondary and tertiary structures of Pgp using infrared attenuated total reflection spectroscopy (Sonveaux et al., 1996). In this work, purified Pgp was functionally reconstituted into liposomes, and the effect of ATP, ATP with VER, VER alone, and ADP on the structure of Pgp was investigated. No effects were observed with VER alone or with ADP. However, the addition of ATP induced a change in the tertiary structure of Pgp.

Sonveaux et al. (1996) used 3 mM ATP versus no ATP as the two experimental states for Pgp. In this study, we have used a running buffer without ATP and one to which we have added the same concentration of ATP (i.e., 3 mM). Thus, the chromatographic results with ATP in the running buffer should reflect the shift in Pgp tertiary structure indicated by Sonveaux et al. (1996). Indeed, the addition of 3 mM ATP to the running buffer increased the retention volume of [^{3}H]CsA from 7.8 to 17.5 ml (Table 2), produced a classical frontal chromatogram for [^{3}H]CsA (Fig. 3C), and permitted the calculation of a K_d value of 62.5 nM (Table 1). These results indicate that the addition of ATP to the running buffer produced a cooperative allosteric interaction that increased the binding affinity of Pgp for CsA. Similar results were obtained in the VBL-CsA binding interaction studies.

The presence of ATP in the running buffer produced the opposite effect on the retention volumes of [^{3}H]VBL and [^{3}H]VER. With [^{3}H]VBL, the addition of 3 mM ATP reduced the observed retention from 32.1 to 8.4 ml (Table 2; Fig. 3), and the retention volume for [^{3}H]VER was reduced from 34.2 to 5.9 ml, with the loss of specific retention in both cases. These results suggest an ATP-induced anticooperative allosteric interaction. Allosterically produced reductions in retention volume can be distinguished from competitive displacements as illustrated by the effect of the addition of VBL on the retention volume of [^{3}H]VBL (Table 2). In this case, the retention volume decreased, but the specific frontal chromatographic curves were retained (data not shown).

Thus, the addition of ATP to the running buffer produced changes in the chromatographic interactions between the ligands and the immobilized Pgp (i.e., specific to nonspecific and vice versa) that are consistent with the changes in the tertiary structure identified by Sonveaux et al. (1996). In this case, the consequence of the change in Pgp tertiary structure was the creation of a specific binding site for CsA. The same change that increased the binding affinity for CsA also altered the site at which VBL binds, decreasing the affinity of Pgp for VBL. The effect of VBL on CsA binding affinity and the effect of ATP on the binding affinities of both VBL and CsA indicate that separate, but closely linked, binding sites for CsA and VBL exist on the Pgp molecule.

The immobilized Pgp liquid chromatographic stationary phase described in this report appears to reproduce Pgp substrate binding as determined by classical filtration binding assays. The observed binding is Pgp-specific, is highly sensitive to changes in the protein's tertiary conformation caused by Pgp interactions with substrates and ATP, and reflects changes occurring in the functional cycle of Pgp. Thus, Pgp-affinity chromatography represents a promising tool for a quick and reproducible evaluation of potential Pgp substrates and/or inhibitors and a useful probe of the transport mechanism. The data obtained through this approach provide new information on Pgp's mechanism of action, including evidence of binding sites for verapamil and for cyclosporins distinct from the ones for Vinca alkaloids. The data directly support a model of Pgp's action where these substrates can bind to distinct, although often allosterically connected, regions.

Acknowledgment

We thank Dr. Yanxiao Zhang for useful discussion.

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Journal of Chromatography B, 739 (2000) 33–37

JOURNAL OF CHROMATOGRAPHY B

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Development of an immobilized P-glycoprotein stationary phase for on-line liquid chromatographic determination of drug-binding affinities

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Abstract

The membrane transporter P-glycoprotein (PGP) has been immobilized on an immobilized artificial membrane (IAM) LC stationary phase. The resulting PGP-IAM phase retained the ability of the native PGP to bind the known PGP-ligand vinblastine. Displacement studies using other known PGP ligands, verapamil and cyclosporin A, demonstrated that there was selective binding between vinblastine and the immobilized PGP transporter. The binding affinity (K_d value) of vinblastine for the PGP-IAM was determined to be 19±20 and 71±11 n*M* on two separate columns. These values are consistent with previously reported values of 9±2, 8±2, and 37±10 n*M*, which were obtained using native membranes. The K_d values obtained on the PGP-IAM for cyclosporin A and verapamil were 492±21 and 172±29 μ*M*, respectively. These results were higher than the corresponding K_d values obtained using native membranes, but the relative affinities vinblastine>cyclosporin A≫verapamil are consistent in both approaches. During several months of experiments and storage, the PGP-IAM was found to be reproducible and stable. The stationary phase appears to be useful in the on-line screening for PGP ligands.

Keywords: Stationary phases, LC; Drug-binding affinities; P-Glycoprotein; Verapamil; Cyclosporin A

1. Introduction

P-glycoprotein (PGP) is a 170–180-kDa membrane transporter [1,2] that acts as an ATP-driven drug efflux pump. The over-expression of PGP has been associated with multidrug resistance (MDR) in tumor cells and the MDR phenotype is a factor in the failure of the chemotherapeutic treatment of breast cancer [3,4]. One approach to the development of therapeutic protocols to overcome MDR in breast cancer patients has concentrated on the inhibition of the PGP-mediated pump. For example, in vitro studies have demonstrated that the presence of verapamil in the incubation media increased the cytotoxicity of vinca alkaloids and anthracycline derivatives in MDR1/PGP tumor cell lines [5]. However, a clinical trial combining verapamil with the vinca alkaloid VP16 and the anthracycline derivative adriamycin was not successful due to the cardiotoxicity of verapamil [6]. Thus, the development of novel agents to reverse MDR1/PGP-mediated drug resistant remains a key objective in breast cancer research.

The functions of PGP have been studied using a

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0378-4347/00/$ – see front matter
PII: S0378-4347(99)00384-9

variety of experimental formats including, detergent solution [7], proteoliposomes [8–12], membrane vesicles [13,14], and native membranes [15–17]. However, the evaluation of ligand-binding parameters and the screening of pools of drug candidates for their PGP binding affinities remain a formidable task.

Our laboratory has recently reported the development of immobilized nicotinic receptor-based liquid chromatographic (LC) stationary phases that can be used for the on-line analysis of drug-receptor interactions [18,19]. In the present work we extend this study to the preparation of PGP-based LC stationary phases for the study of drug–PGP interactions. One PGP-based stationary phase was prepared by embedding PGP in the phospholipid monolayer of an immobilized artificial membrane (IAM) HPLC stationary phase [20] creating the PGP-IAM. In a second approach, PGP was also reconstituted into the phospholipid bilayer of liposomes that were immobilized on Superdex 200 gel beads by using freeze-thawing methods (PGP-LIP). The latter approach was originally developed for the immobilization of liposomes or liposomes containing human red cell glucose transporter in chromatographic stationary phase [21,22].

In this study, the PGP binding affinities of vinblastine, cyclosporin A and verapamil were assessed using the PGP-IAM stationary phase and frontal chromatographic techniques. The rank order of the calculated K_d values, i.e. highest affinity to lowest affinity, were consistent with previously reported values [15]. The PGP-IAM was stable, reproducible and appears to be a useful addition to the study of PGP–ligand interactions and for the rapid on-line screening of new agents for the treatment of MDR1/PGP resistant tumors.

2. Experimental

2.1. Chemicals

L-α-Lecithin [20% phosphatidylcholine (PC)], *E. coli* bulk phospholipid and L-α-phosphatidylserine (PS) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). IAM.PC particles were obtained from Regis Chemical Co. (Morton Grove, IL, USA). Superdex 200® prep grade (a gel filtration media with a unique composite matrix of dextran and agarose), Sephadex G50 medium and glass column (HR5/5 and HR 5/10) were purchased from Amersham Pharmarcia Biotech (Uppsala, Sweden). [^{3}H]Vinblastine sulphate was from Amersham Life Science Products (Boston, MA, USA). Cyclosporin A, octyl-β-D-glucopyranoside, leupeptin, pepstain A, glycerol, benzamidine, cholesterol (>99%), and other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Scintillation liquid (Flo-Scint V) was purchased from Packard Instruments (Meriden, CT, USA).

2.2. Immobilization of PGP in IAM particles

Cultured MDA435/LCC6^{MDR1} cells which over express the MDR1 gene and PGP [23] were used as the source of the PGP. About 2×10^6 cells were harvested in 18 ml of 50 m*M* Tris–HCl buffer [50 m*M*, pH 7.4] {Buffer A} containing 50 m*M* NaCl, 2 μ*M* leupeptin and 4 μ*M* pepstatin A. The mixture was homogenized for 2×20 s with a Brinkmann Polytron homogenizer, the homogenates were centrifuged at 35 000 *g* for 10 min, the supernatant was discarded and the pellets resuspended in 6 ml solubilization solution [Buffer A containing 250 m*M* NaCl, 0.5% CHAPS {3-[(3-cholamidopropyl)-dimethylammonio]-1-propane-sulfonate}, 2 m*M* DTT (dithiothreitol), 5% glycerol] for 2 h at 4°C. Dried IAM.PC particles (100 mg) were suspend in the PGP-CHAPS solution (7 ml) and stirred for 1 h at 4°C. The mixture was then dialyzed against dialysis buffer [150 m*M* NaCl, 10 m*M* Tris–HCl buffer, pH 7.5, 1 m*M* EDTA (ethylenediamine-teraacetic acid), 1 m*M* benzamidine] for 36 h at 4°C. The obtained PGP-IAM particles were washed with Buffer A by centrifugation and packed in a glass column.

2.3. Reconstitution and immobilization of PGP in Superdex 200 gel beads

The membrane pellet obtained as described above was suspended in 4 ml solubilization solution [50 m*M* Tris–HCl, pH 7.5 containing 1.4% octyl-β-D-glucopyranoside, 20% glycerol, 1 m*M* DTT, 1 m*M* benzamidine and 0.4% phospholipid: *E. coli* bulk phospholipid–PC–PS–cholesterol (60:17.5:10:12.5,

v/v)] by stirring at 0°C for 40 min. Non-soluble material was removed as a pellet by centrifugation. The supernatant was applied on to a Sephadex G50 column (1×80 cm) which equilibrated with elution buffer [150 m*M* NaCl, 10 m*M* Tris–HCl, 1 m*M* EDTA, 1 m*M* Benzamidine]. The liposome fractions (18 ml) were collected and concentrated to 1 ml. The concentrated liposome solution was mixed with 50 mg dried Superdex 200 and kept in room temperature for 2 h. The mixture of liposome and Superdex 200 was frozen at −75°C for 10 min and thawed at 25°C for 10 min. The freezing and thawing was repeated once. The non-immobilized liposomes were removed by washing the pellet with Buffer A until the supernatant was clear in centrifugation. The obtained PGP-Superdex gel beads were packed in a chromatographic column (0.5 cm I.D.).

2.4. Frontal chromatographic analysis of binding affinity of drugs with PGP-IAM stationary phase

The PGP-IAM column was placed in a standard HPLC system and equilibrated with Buffer A. [^{3}H]Vinblastine (10–45 ml of a 0.5 or 1 n*M* solution in Buffer A in the absence or presence of cold vinblastine) was applied to the column and an elution profile with frontal and plateau (see an example of the elution profiles in Fig. 1) is recorded by an on-line flow scintillation detector (Radiomatic™ 525 TR, Packard Instruments). The radioactive signal (CPM) in the outlet eluate were recorded in 6-s intervals, summed up in 1-min intervals and smoothed with a ten-point moving average using the Microsoft Excel program. The retention volumes of [^{3}H]vinblastine (0.5 n*M*) in the absence and presence in different concentration of drugs in the mobile phase were taken as the elution volume corresponding to the half height of plateau. The flow-rate, 0.4 ml/min, was used in all runs of chromatography. Vinblastine and doxorubicin were solubilized in Buffer A, verapamil was solubilized in Buffer A with 20% ethanol and the final concentration of ethanol was adjusted to 1.6% and cyclosporin A was solubilized in Buffer A with 1.6% ethanol.

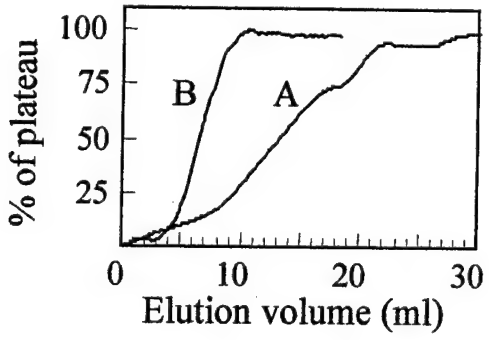

Fig. 1. Elution profiles of [^{3}H]VB [1 nM] in frontal chromatography based on the PGP-IAM column (0.5×0.8 cm) in the absence (profile A) and presence of doxorubicin [200 nM] (profile B) in the mobile phase: Tris–HCl buffer [50 m*M*, pH 7.4]. Flow rate: 0.4 ml/min.

2.5. Bicinchoninic acid (BCA) protein assay

The PGP-IAM particles, PGP-Superdex 200 gel beads, IAM particles, Superdex 200 gel beads were collected. The samples were diluted with 0.1 m of NaOH to 2 ml. A protein standard (0.2–25 μg protein in 50 μl) was prepared with albumin standard (Pierce) and amount of proteins were detected using Pierce BCA protein assay kit (BCA is a trademark of Pierce for protein assays using bicinchoninic acid and the reagents contain sodium carbonate, sodium bicarbonate, BCA detection reagent, and sodium tartrate in 0.1 m sodium hydroxide). The standards and samples (50 μl each) were added to triplicate wells in a 96-well plate, 20 ml of reagent A was mixed with 0.4 ml of reagent B and a 200-μl aliquots of the resulting BCA reagents (A+B) were added to each well. The plate was incubated for 2 h at room temperature and read in a spectrophotometer at 570 nm using Softmax program for the calculation of protein amount.

3. Results and discussion

Protein assay showed that for 1 ml of bed volume about 170 mg proteins were immobilized in IAM particles and about 10 mg proteins were immobilized in Superdex 200 gel beads. On a PGP-IAM column (0.5×0.8 cm), [^{3}H]vinblastine (1 n*M*) was retarded and showed a frontal profile with retention volume 13.3 ml (Fig. 1, profile A). When a known displacer, doxorubicin (200 n*M*), was included in the mobile phase, the retention volume of [^{3}H]vinblastine [1

nM] was decrease from 13.3 to 6.5 ml (Fig. 1, profile B). This displacement experiment indicated that the specific binding activity of PGP was retained after immobilization.

The K_d value of vinblastine (K_{VB}) and the number of the active and available binding sites (B_{max}) of immobilized PGP were calculated from the retention volumes, V, of [^{3}H]vinblastine at the different concentration in frontal chromatography according to Eq. (1):

$$[VB](V-V_{min}) = B_{max}[VB]/(K_{VB}+[VB]) \quad (1)$$

This equation was adapted from the rectangular hyperbola equation [24] using a previously described approach (see Eq. (3) in Ref. [25]). Using this approach, K_d and B_{max} can be calculated by the plotting $[VB](V-V_{min})$ vs. [VB] or by one binding site nonlinear regression with program Prism (GraphPad Software), taking [VB] as X values and $[VB](V-V_{min})$ as Y values. V_{min} is the elution volume of vinblastine [0.5 nM] when the specific interaction is completely suppressed. V_{min} can be taken approximately as the retention volume in the presence of high concentration of vinblastine. When a competitive displacer (drug) was included in the mobile phase, V_{min} value can be calculated more precisely by Eq. (2) (below).

The calculated K_d value for vinblastine determined on the initial PGP-IAM column (0.5×0.8 cm) in Buffer A was 19±20 nM with B_{max} 546±60 nmol and on a second PGP-IAM column (0.5×1.5 cm) was 71±11 nM with B_{max} 1073±57 nmol. The retention volume of vinblastine [0.5 nM] on the second PGP-IAM column was 25.0±1.0 ml during the initial chromatographic run and 24.5±0.8 ml after over 1 month of use at room temperature. These results demonstrate that the PGP-IAM is reproducible and stable.

The addition of ethanol [1.6%] was necessary for the solubilization of the hydrophobic drugs in the mobile phase. When 1.6% ethanol was included in the mobile phase the retention volume of vinblastine [0.5 nM] was decreased from 25.0±1.0 to 15±1 ml. Verapamil and cyclosporin A were still able to displace vinblastine and ethanol appears to predominately affect the non-specific retention interactions.

The retention volumes, V, of [^{3}H]vinblastine [0.5 nM] measured in the absence or presence of verapamil and cyclosporin A, respectively, at different concentrations, [drug], were used to calculate the K_{drug}, according to Eq. (2) [18]:

$$(V_{max}-V)^{-1} = (1+[VB]K_{VB})(V_{min}B_{max}K_{VB})^{-1} + (1+[VB]K_{VB})^2(V_{min}B_{max}K_{VB}K_{drug})^{-1}[drug]^{-1} \quad (2)$$

By plotting $(V_{max}-V)^{-1}$ vs. $[drug]^{-1}$, V_{min} was obtained when [drug] was extrapolating to infinity, and K_{drug} or B_{max} for drugs were calculated from the ratio between the slop and the ordinate intercept, which equals $(1+[VB]K_{VB})/K_{drug}$. The obtained K_d values of vinblastine, verapamil and cyclosporine were presented in Table 1. The mean and deviations of the K_d values were obtained using the retention volumes from two runs.

The K_d value of vinblastine measured from two PGP-IAM columns is 19±20 and 71±11 nM. These values are consistent with previously reported values of 9±2 nM [15], 8±2 nM [16] and 37±10 nM [17] that were obtained using native membranes. In another study, the K_d value of vinblastine was found to be 36±55 nM in native membranes and 130±9 nM after the PGP were solubilized in detergent [9]. The authors concluded that solubilization has altered the PGP phospholipid environment reducing its specific capacity.

The K_d values obtained for cyclosporin A (492±21 nM) and verapamil (172±29 μM) are higher than the previously reported values of 17±2 nM [15], 18±3 nM [17] for cyclosporin A and 600±180 nM [15], 452±50 nM [17] for verapamil, respectively. These results may be due to the fact that PGP has at least two allosterically coupled binding sites [17] and that the solubilization of the protein before immobilization on the IAM support

Table 1
K_d values calculated using frontal chromatography on an PGP-IAM column

Drugs	K_d
Vinblastine	71±11 nM
Cyclosporine A	492±21 nM
Verapamil	172±29 μM

may have altered the configuration of one or more of these sites. This possibility is under investigation.

Although the absolute K_d values determined on the PGP-IAM are higher than those obtained by other methods, the relative affinities are the same. This demonstrates that the method previously developed for immobilization of nicotinic receptors in LC stationary phase also can be used to immobilize PGP for study of interaction between PGP and drugs.

The methods to reconstitute into liposomes and immobilize PGP-liposomes in Superdex 200 gel beads were also included in this manuscript as an alternative for the preparation of PGP-LC stationary phase. PGP reconstituted in the phospholipid bilayer of the immobilized proteoliposomes also displayed a similar binding activity as the PGP immobilized in IAM particles (data not shown). Since immobilized PGP-Superdex 200 did not show obvious advantages over PGP-IAM in the study of drug binding on PGP, additional experiments are being performed on PGP-Superdex 200 column and will be reported at a future date.

Acknowledgements

This work was supported by NIH grant 1R426M56591-02 (IWW) and USAMRMCRP9550649 (RC).

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Pergamon

European Journal of Cancer, Vol. 35, No. 10, pp. 1541–1545, 1999

Printed in Great Britain
0959-8049/99/$ - see front matter

PII: S0959-8049(99)00180-X

Original Paper

Inhibition of P-glycoprotein Activity and Reversal of Multidrug Resistance *In Vitro* by Rosemary Extract

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The transmembrane transport pump P-glycoprotein (Pgp) causes the efflux of chemotherapeutic agents from cells and is believed to be an important mechanism in multidrug resistance (MDR) in mammary tumours. In the present study we demonstrate that an extract of the common dietary herb rosemary (*Rosemarinus officinalis* Labiatae), increases the intracellular accumulation of commonly used chemotherapeutic agents, including doxorubicin (DOX) and vinblastine (VIN), in drug-resistant MCF-7 human breast cancer cells which express Pgp. Rosemary extract (RE) inhibits the efflux of DOX and VIN, which are known to be substrates of Pgp, but does not affect accumulation or efflux of DOX in wild type MCF-7 cells, which lack Pgp. Treatment of drug-resistant cells with RE increases their sensitivity to DOX, which is consistent with an increased intracellular accumulation of the drug. RE blocks the binding of the VIN analogue azidopine to Pgp. Thus, it appears that RE directly inhibits Pgp activity by inhibiting the binding of drugs to Pgp. © **1999 Elsevier Science Ltd. All rights reserved.**

Key words: rosemary, P-glycoprotein, multidrug resistance, breast cancer, doxorubicin
Eur J Cancer, Vol. 35, No. 10, pp. 1541–1545, 1999

INTRODUCTION

MULTIDRUG RESISTANCE (MDR), the cross-resistance of tumour cells to a variety of structurally and functionally unrelated anticancer drugs, is a major obstacle in cancer treatment [1]. Although there are several mechanisms which confer MDR, one that is often overexpressed in mammary tumours following drug treatment is the 170 kD plasma membrane-associated glycoprotein (Pgp). Pgp acts as an energy-dependent drug efflux pump that decreases intracellular drug accumulation, thereby decreasing the effectiveness of many chemotherapeutic agents [2]. Since Pgp can confer MDR on tumour cells, the development of agents which inhibit the Pgp-mediated efflux of drugs, and thus reverse MDR, has been intensively pursued [3]. A broad range of compounds have been identified that are able to reverse MDR by blocking Pgp activity *in vitro*. However, they have not been widely used in the treatment of cancer patients because the doses required to reverse MDR resistance are either toxic or not clinically achievable [4]. A 'second generation' of MDR reversal agents, such as the cyclosporin derivative PSC 833 [5] has recently been identified and are currently undergoing clinical trial.

Our laboratory has previously investigated the effect of several natural, plant-derived chemicals on the activity of Pgp. Several members of the flavonoids, a diverse group of structurally related polyphenolic compounds widely distributed in plants, were shown to increase the activity of Pgp towards carcinogens such as dimethylbenz[a]anthracene [6]. Since Pgp is expressed in normal tissues, we hypothesised that these flavonoids may have chemopreventive activity against environmental carcinogens by increasing the cellular efflux of these compounds from cells via Pgp. However, in a chemotherapeutic setting, an increase in Pgp activity would be counterproductive, and, indeed, we found that these flavonoids also increase the Pgp-mediated efflux of the chemotherapeutic drug doxorubicin (DOX) [7]. We, therefore, investigated other phytochemicals which might inhibit Pgp activity and reverse MDR. Extracts of rosemary (*Rosemarinus officinalis* Labiatae), a commonly used herb, has been previously shown to have potent chemopreventive activity *in vivo*

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Received 11 Mar. 1999; revised 18 Jun. 1999; accepted 22 Jun. 1999.

[8,9]. As a result, rosemary extract (RE) has been well studied in animal models and has been shown to be antimutagenic [10,11] and nontoxic [12]. With RE already established as an effective chemopreventive agent and nontoxic in animal models, we investigated its effect on Pgp activity and MDR. In this report we demonstrate, for the first time, that RE inhibits Pgp-mediated drug efflux, resulting in an increase in the intracellular accumulation and cytotoxicity of chemotherapeutic drugs in drug-resistant human breast cancer cells *in vitro*.

MATERIALS AND METHODS

Materials

DOX, verapamil, vinblastine (VIN), kaempferol and dimethylsulphoxide (DMSO) were purchased from Sigma Chemical Company (St Louis, Missouri, U.S.A.). The RE compounds carnosic acid, carnosol and rosemarinic acid were gifts of Kelsec, Inc. (Kalamazoo, Michigan, U.S.A.). All radio-labelled compounds were purchased from Amersham (Arlington Heights, Illinois, U.S.A.).

Preparation of RE

RE was prepared according to the method of Wu and colleagues [13]. Briefly, 1 kg of powdered rosemary leaves were extracted with 6 l of methanol at 60°C for 2 h. The mixture was filtered and re-extracted with 4 l of methanol. The combined filtrate was bleached with 200 g of active charcoal, filtered and concentrated by rotary evaporation to 900 ml before filtering to remove precipitates. The filtrate was rotary evaporated to dryness to produce the RE. Purification of RE by this method gave a yield of 77 g from 1 kg of ground RE. RE was dissolved in DMSO with the final DMSO concentration used for experimentation adjusted to 0.1% (v/v) in media.

Cell culture

Three different MCF-7 human breast cancer cell lines were used in this study: the wild-type (WT), which does not express Pgp; R65, an MCF-7-derived line which expresses Pgp and has acquired resistance to DOX and VIN [14]; and Clone 10.9, a stable *MDR1*-transfected MCF-7 line with resistance to DOX and colchicine [15]. Cells were maintained in RPMI 1640 (BioFluids, Rockville, Maryland, U.S.A.) supplemented with 10% (v/v) fetal bovine serum (Life Technologies, Rockville, Maryland, U.S.A.) and 2 mM L-glutamine (BioFluids) at 37°C in a humid atmosphere containing 5% CO_2. Cells were passed weekly using 0.05% trypsin/0.01% ethylenediaminetetra-acetic acid (EDTA).

Intracellular drug accumulation

WT and R65 cells were plated out at 200 000 cells per well and Clone 10.9 cells were plated out at 320 000 cells per well in 6-well plates and allowed to grow to confluence. Cells were pretreated with DMSO (control) or 82 μg of RE per ml of culture medium for 30 min, then exposed to 0.01 μCi [^{14}C]DOX/ml, 0.05 μCi [^{3}H]paclitaxel/ml, or 0.05 μCi [^{3}H]VIN/ml for 60 min. The medium was removed and the plates were washed extensively with phosphate-buffered saline (PBS). The cells were then trypsinised (0.25% trypsin/3 mM EDTA), transferred to scintillation vials to which 20 ml of Aquasol scintillation fluid (Beckman, Palo Alto, California, U.S.A.) was added, and the amount of intracellular radioactivity was determined.

Measurement of Pgp-mediated drug efflux

WT, R65, and Clone 10.9 cells were plated out as described for drug accumulation experiments and allowed to grow to confluence. Cells were pretreated with DMSO (control), 16.5 μg/ml or 85 μg/ml of RE (Figure 1), or 50 μM (16.5 to 18.0 μg/ml) of purified RE components for 30 min, then exposed to 0.01 μCi [^{14}C]DOX/ml for 60 min at 37°C. The medium was removed and the plates washed extensively with PBS. Fresh medium was added and the cells were incubated for 30 min at 37°C. The medium was removed, and the amount of [^{14}C]DOX in the medium that had been effluxed into the medium was measured by scintillation counting. The cells were trypsinised and also counted. Data are expressed as the per cent of total [^{14}C]DOX present in the medium.

Measurement of DOX cytotoxicity

WT and R65 cells were plated out at 20 000 cells per well and Clone 10.9 cells were plated out at 26 000 cells per well in 24-well plates. After 24 h, medium containing DMSO (control) or 16.5 μg of RE per ml of culture medium in the presence of various concentrations of DOX was added. The cells were incubated for 4 days at 37°C, and cell growth was assessed by sulphorhodamine assay [16].

Plasma membrane preparation and [^{3}H]azidopine labelling

Plasma membranes for azidopine labelling studies were prepared from confluent R65 cell cultures. Cells were scraped from the tissue culture flasks and pelleted by centrifugation. The cell pellet was resuspended in buffer consisting of 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, and Complete protease inhibitor cocktail (Beckman) and sonicated. The sonicate was centrifuged at 120 000*g*, 4°C, for 60 min in an Optima XL-90 Ultracentrifuge (Beckman). The membrane pellet was resuspended in 50 mM Tris-HCl, pH 7.4; 16% (w/v) sucrose with Complete, and layered on to 31% (w/v) sucrose for centrifugation at 70 000*g*, 4°C, for 18 h. The partially purified Pgp membranes were dialysed overnight at 4°C against 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, and Complete prior to use. Photoaffinity labelling of the partially purified membranes with [^{3}H]azidopine was carried out as previously described [14]. DOX, verapamil, kaempferol, and

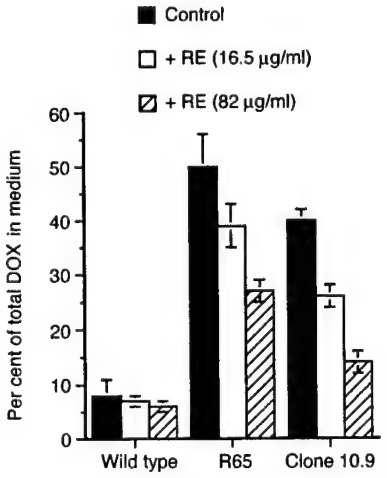

Figure 1. Effect of RE on the efflux of [^{14}C]DOX. $n = 3 \pm$ standard deviation (S.D.). There was a significant decrease in [^{14}C]DOX efflux in R65 and Clone 10.9 cells treated with RE, but no difference in WT cells ($P < 0.05$).

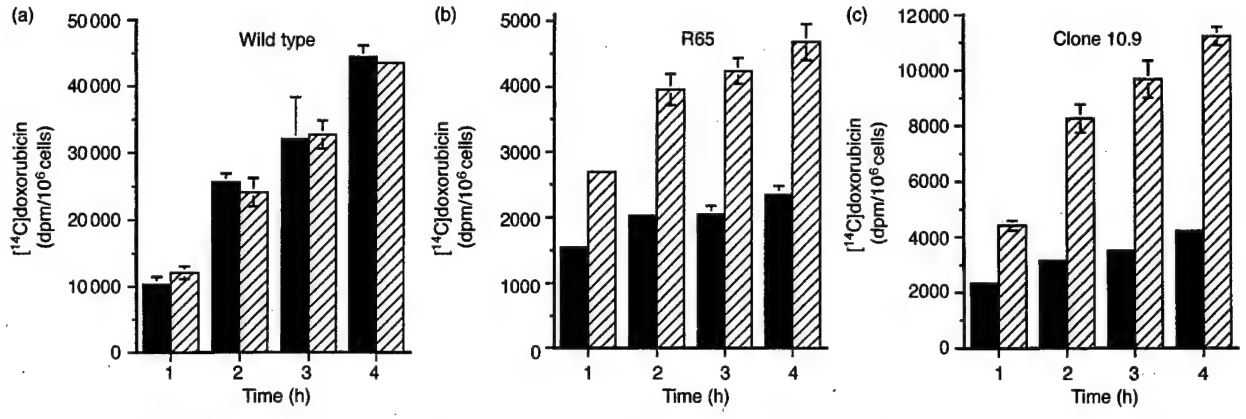

Figure 2. Effect of RE on the accumulation of [^{14}C]DOX in MCF-7 cells. $n = 3 \pm$ standard deviation (S.D.). There was a significant increase in [^{14}C]DOX accumulation in R65 and Clone 10.9 cells treated with RE compared with controls at every time point examined ($P < 0.05$), but no difference in WT cells. ■ control (DMSO), RE treated.

VIN used in the [^{3}H]azidopine labelling were all at a concentration of 0.2 μM, which represents a 330-fold excess over the amount of [^{3}H]azidopine. RE was used at 0.066 μg/ml.

Statistical analysis

Statistical analyses were performed using StatView Statistical Analysis software (SAS Institute; San Francisco, California, U.S.A.). Differences between group mean values were determined by a one-factor analysis of variance (ANOVA), followed by Fisher PSLD *post-hoc* analysis for pairwise comparison of means.

RESULTS

Modulation of intracellular drug accumulation by RE

We have previously developed and characterised MDR cell lines derived from human breast cancer MCF-7 cells by continuous exposure to DOX [6, 14]. These cells overexpress Pgp, resulting in a 65-fold increase in DOX resistance compared to WT cells and are, therefore, designated as R65. WT and R65 cell lines, as well as Clone 10.9, a MCF-7 cell line which expresses Pgp as a result of a stable transfection with the *MDR1* gene, were used in the present study to examine the effect of RE on drug accumulation, Pgp activity, and MDR.

We examined the effect of RE on the intracellular accumulation of several commonly used chemotherapeutic agents.

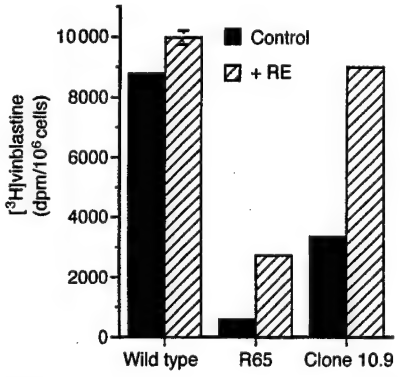

Figure 3. Effect of RE on the accumulation of [^{3}H]VIN. The accumulation of [^{3}H]VIN in WT, R65, and Clone 10.9 cells in the presence or absence of RE (82 μg/ml) was determined. $n = 3 \pm$ standard deviation (S.D.). There was a significant increase in [^{3}H]vinblastine accumulation in R65 and Clone 10.9 cells in the presence of RE ($P < 0.05$).

As seen in Figure 2, incubation of WT cells with [^{14}C]DOX resulted in a time-dependent accumulation of DOX. This was not affected by exposure to RE. In R65 and Clone 10.9 cells there was no significant accumulation of [^{14}C]DOX in DMSO controls over the time period studied, but exposure to RE resulted in a significant increase ($P < 0.05$) in DOX accumulation in R65 and Clone 10.9 cells compared with controls.

RE treatment also caused an increase in the cellular accumulation of [^{3}H]VIN in R65 cells and Clone 10.9 cells, but did not affect [^{3}H]VIN accumulation in WT cells (Figure 3). RE also caused a significant increase in the amount of [^{3}H]paclitaxel accumulation in R65 cells (data not shown).

Effect of RE on DOX efflux

The effect of RE on the Pgp-mediated efflux of DOX was examined. As shown in Figure 1, RE caused a decrease in the amount of DOX effluxed from R65 and Clone 10.9 cells, whilst having no effect on efflux in WT cells. Similarly, in normal human breast cells (Clonetics, San Diego, California, U.S.A.), which have little or no Pgp expression, there was no change in the accumulation or efflux of DOX in the presence of RE (data not shown).

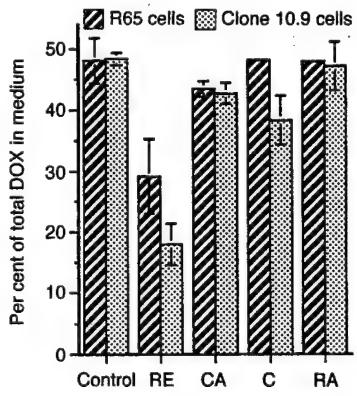

Figure 4. Effect of purified RE components on [^{14}C]DOX efflux. The efflux of [^{14}C]DOX in the presence of 50 μM (approximately 16.5 to 18 μg/ml) of the RE components carnosic acid (CA), carnosol (C), rosemarinic acid (RA), or 82 μg/ml RE was determined in R65 (striped bars) and Clone 10.9 cells (dotted bars). $n = 3 \pm$ standard deviation (S.D.). There was no significant difference in efflux in R65 or Clone 10.9 cells with any treatment except RE ($P < 0.05$).

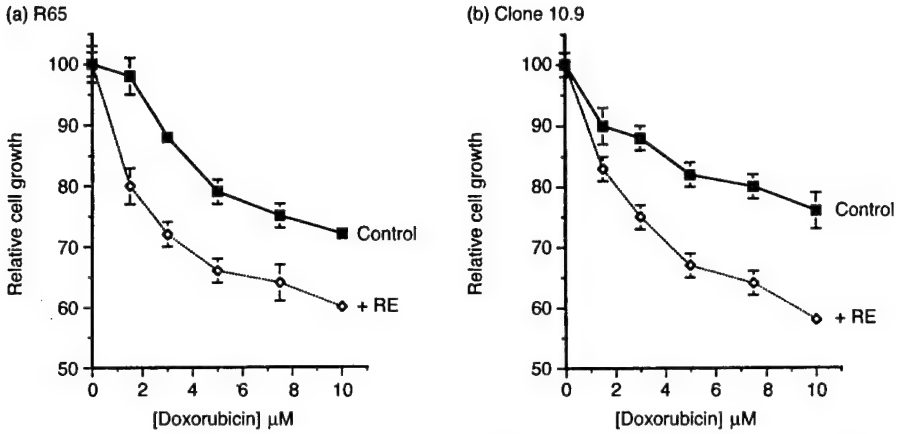

Figure 5. Effect of RE on the cytotoxicity of DOX. (a) R65 and (b) Clone 10.9 cells were grown in the presence of DMSO (control) or RE (16.5 μg/ml) in the presence of different concentrations of DOX. The amount of cell growth was determined after 3 days by sulphorhodamine. RE at this concentration had no significant inhibitory activity on cell growth by itself. $n = 3 \pm$ standard deviation (S.D.). There was a significant difference in cell growth in RE-treated cultures compared with controls in both cell lines ($P < 0.05$).

The RE prepared for these experiments is a complex mixture of compounds. Previous experiments on the antioxidant properties of RE led to the purification of the active antioxidant components carnosol, carnosic acid, and rosemarinic acid [13, 17]. To determine if any of these substances were the active component responsible for modulation of Pgp activity, we examined their effect on DOX efflux in R65 and Clone 10.9 cells. There was no significant inhibition of DOX efflux in R65 or Clone 10.9 cells treated with carnosic acid, carnosol, or rosemarinic acid (Figure 4).

Effect of RE on DOX cytotoxicity

Because RE increased the intracellular accumulation of DOX, we examined its effect on DOX-induced cytotoxicity as measured by inhibition of cell growth. Incubation of WT cells with increasing amounts of DOX resulted in a decrease in cell growth that was not affected by incubation with RE (data not shown). In R65 and Clone 10.9 cells, co-incubation of DOX with RE resulted in a significant increase in the cytotoxicity of DOX (Figure 5a and b). RE also decreased the IC_{50} of VIN in R65 cells (data not shown). A lower RE concentration was used in cytotoxicity experiments than in accumulation and efflux experiments because RE itself inhibited cells growth ($IC_{50} = 30$ μg/ml for all cell lines; data not shown). Higher concentrations of RE which lead to even greater accumulation of DOX could not, therefore, be tested with regard to cytotoxicity. The higher concentrations used in Figures 1–3 did not affect cell viability because the incubation time was short and the cells were confluent.

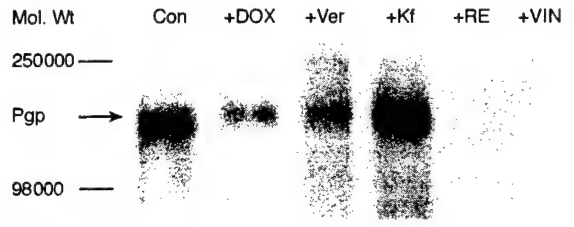

Figure 6. Photoaffinity labelling of Pgp with [^{3}H]azidopine in the presence of RE or other effectors of Pgp. Partially purified plasma membranes from R65 cells containing Pgp were subjected to photoaffinity labelling with the VIN analogue [^{3}H]azidopine in the presence of DMSO (Con), or a 330-fold excess of DOX, verapamil (Ver), kaempferol (kf), RE , or VIN.

Effect of RE on photoaffinity labelling of Pgp

Substrates and reversal agents of Pgp are known to inhibit photoaffinity labelling of Pgp by the VIN analogue [^{3}H]azidopine by competing with azidopine for the substrate binding site(s) of Pgp. In order to determine whether RE interacts directly with Pgp binding site(s), we examined photoaffinity labelling of Pgp in the presence of RE. The presence of a 330-fold excess of RE (compared with [^{3}H]azidopine) completely abolished binding of [^{3}H]azidopine to Pgp (Figure 6). RE was as effective as VIN at this concentration at inhibiting labelling, and more effective than DOX or the Pgp reversal agent verapamil. Kaempferol, a flavonoid which we have previously shown to increase Pgp activity [6, 14], increased [^{3}H]azidopine labelling.

DISCUSSION

The intracellular level of some chemotherapeutic drugs is decreased by the activity of Pgp, the ATP-dependent efflux pump encoded by the *MDR1* gene, which is up-regulated in many drug-resistant tumour cells [1, 18]. Much attention has, therefore, been devoted to the development of agents which inhibit Pgp activity and reverse MDR. Unfortunately, the most effective Pgp inhibitors *in vitro*, such as verapamil, have proven to be too toxic to be used clinically. A readily available, nontoxic Pgp inhibitor may prove to be efficacious when administered in combination with commonly used chemotherapeutic pleiotropic drugs whose effectiveness is compromised by Pgp, such as DOX, VIN, and paclitaxel. Therefore, the effects of many plant-derived compounds on Pgp were investigated. These phytochemicals have the advantage of being natural dietary compounds that are nontoxic in animals. In the present study we investigated the effects of RE on drug accumulation and Pgp activity *in vitro*. RE, a complex mixture of chemicals extracted from rosemary, has shown much promise in the prevention of chemically-induced carcinogenesis in animal models [8, 9, 17]. We carried out these experiments in a DOX-resistant MCF-7 cell line which expresses high levels of Pgp compared with WT cells. These cells were developed by continuous exposure to DOX over a period of months. To confirm these results, we also examined the effect of RE in Clone 10.9 cells, an MCF-7 cell line which is stably transfected with *MDR1*.

RE caused a substantial increase in the accumulation of DOX or VIN in cells expressing Pgp, and inhibited the efflux of DOX, but had no effect on WT cells which lack Pgp. RE also caused an increase in paclitaxel accumulation in these cells (data not shown). Since these drugs are known to be substrates for Pgp, we concluded that RE modulates intracellular drug levels by inhibiting Pgp. In agreement with these data, RE also increased the cytotoxicity of DOX in Pgp-expressing cell lines, but not WT cells. Exposure to RE also increased the cytotoxicity of VIN (data not shown). This demonstrates that RE can partially reverse MDR in cells which express Pgp.

Since the time of exposure of cells to RE in these experiments was short (1 to 4 h), it is unlikely that RE acts by down-regulating *MDR1* transcription and, therefore, reducing the amount of cellular Pgp. Nevertheless, we examined the effect of RE on the expression of Pgp at the protein (Western) and mRNA (Northern) levels. There was no difference in Pgp expression in any of the three cell lines used in this study when treated with RE for a period of 4 days (data not shown). Taken together, these data indicate that RE increases intracellular drug levels by modulating Pgp activity, not expression.

Several laboratories have isolated constituents present in RE that are primarily responsible for the antioxidant activity of RE. To attempt to identify the constituent of RE responsible for Pgp inhibition, we examined the effect of several of these phytochemicals on DOX efflux. The concentration of carnosic acid and carnosol in RE prepared by the method of Wu and colleagues [13] is approximately 7.5% each. The concentration of carnosic acid or carnosol in our assays of drug accumulation or efflux is, therefore, approximately 6 μg/ml. Neither carnosic acid, carnosol, nor rosemarinic acid significantly affected DOX efflux in R65 or Clone 10.9 cells, even at a concentration of 16.5 μg/ml (Figure 4). Thus, carnosic acid, carnosol, and rosemarinic acid do not contribute to the inhibitory effect of RE. Some other of the thousands of phytochemicals present in RE, or a combination of phytochemicals, must be responsible for this activity, but the 'active ingredient(s)' remains to be determined.

In order to determine the mechanism of RE's activity, we employed [^{3}H]azidopine photoaffinity labelling. [^{3}H]azidopine, a VIN analogue, binds to the substrate binding site(s) of Pgp. Inhibition of labelling by a compound indicates that the compound directly competes for the substrate binding site(s), thus blocking the binding of azidopine. In this manner, the Pgp substrates DOX and VIN, or the Pgp inhibitor verapamil, inhibit azidopine labelling of Pgp in partially purified plasma membranes (Figure 6). RE completely abolished photoaffinity labelling, indicating that RE directly binds to Pgp at the binding site(s). Thus, this suggests that the mechanism of RE's inhibitory activity is through a competitive inhibition of substrate binding. Whether the active component of RE itself undergoes transport by Pgp is unknown.

RE, unlike other Pgp inhibitors, is not only plentiful and inexpensive to prepare, but relatively nontoxic. Although the present experiments demonstrate that RE is an effective inhibitor of Pgp activity *in vitro*, animal experimentation is required to determine if RE has potential as an effective and safe 'chemosensitiser' for treating cancers expressing Pgp-mediated MDR. Furthermore, despite intense interest in rosemary extract as an inhibitor of experimental carcinogenesis in animal studies, the physiologically relevant concentrations attainable in humans have not been reported. It is important to consider that the levels of MDR and Pgp expression in the cell lines used in this study are much greater than that developed in human tumour cells. Thus, RE may be an even more effective MDR reversal agent in actual tumours. The effect of RE on other drug efflux mechanisms such as the multidrug resistance protein [19] is currently under investigation.

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Acknowledgements—The authors wish to thank Mr P.J. Daschner and Mr F.M. Segreti for preparing the rosemary extract. We are grateful to D. Berdahl and Kalsec, Inc., Kalamazoo, Michigan, U.S.A. for the gifts of carnosol, carnosic acid, and rosemarinic acid.

ARTICLES

Multidrug Resistance in Breast Cancer: a Meta-analysis of MDR1/gp170 Expression and Its Possible Functional Significance

*Bruce J. Trock, Fabio Leonessa, Robert Clarke**

Background: **P-glycoprotein (gp170; encoded by the MDR1 gene [also known as PGY1]) is a membrane protein capable of exporting a variety of anticancer drugs from cells. MDR1/gp170 expression has been studied in breast cancer, but the prevalence of this expression and its role in breast tumor drug resistance are unclear.** ***Purpose:*** **We conducted a critical review and meta-analysis of studies examining MDR1/gp170 expression in breast cancer to estimate the likely prevalence and clinical relevance of this expression. We also explored reasons for differences in the findings from individual studies.** ***Methods:*** **Published papers on MDR1/gp170 expression in breast cancer were identified by searching several literature databases and reviewing the bibliographies of identified papers. Variability across the studies in the proportion of tumors expressing MDR1/gp170 was assessed by use of chi-squared tests of homogeneity, weighted means, and weighted linear regression. Pooled relative risks (RRs) for the association between the induction of MDR1/gp170 expression and prior chemotherapy and associations between MDR1/gp170 expression and several clinical outcomes were estimated by use of Mantel–Haenszel methods. Heterogeneity among the pooled RRs was explored by use of chi-squared tests. Reported *P* values are two-sided.** ***Results:*** **Thirty-one studies were identified and evaluated. The proportion of breast tumors expressing MDR1/gp170 in all of the studies was 41.2%, but there was substantial heterogeneity in the values across individual studies ($P<.0001$). Regression analyses demonstrated that a considerable portion of the observed heterogeneity was a consequence of the change, over time, from RNA hybridization-based assays to immunohistochemistry-based assays of MDR1/gp170 expression. Measuring MDR1/gp170 expression before versus after chemotherapy and use of cytotoxic drugs that are not substrates for gp170 also contributed to the heterogeneity. Treatment with chemotherapeutic drugs or hormonal agents was associated with an increase in the proportion of tumors expressing MDR1/gp170 (RR = 1.77; 95% confidence interval [CI] = 1.46-2.15). Patients with tumors expressing MDR1/gp170 were three times more likely to fail to respond to chemotherapy than patients whose tumors were MDR1/gp170 negative (RR = 3.21; 95% CI = 2.28-4.51); this RR increased to 4.19 (95% CI = 2.71-6.47) when considering only patients whose tumor expression of MDR1/gp170 was measured after chemotherapy. MDR1/gp170 expression was not associated with lymph node metastases, estrogen receptor status, tumor size, tumor grade, or tumor histology.** ***Conclusions and Implications:*** **MDR1/gp170 expression in breast tumors is associated with treatment and with a poor response to chemotherapy. The data are consistent with a contributory role for MDR1/gp170 in the multidrug resistance in some breast tumors. [J Natl Cancer Inst 1997;89:917-31]**

Breast cancer is often considered to be one of the more chemoresponsive solid tumors. Many structurally diverse cytotoxic drugs, when administered either as single agents or in combination, can induce remissions in previously untreated breast cancer patients (*1*). While the overall response rate can be high, the duration of response is relatively short (*2*), and most of the initially responsive breast tumors acquire a multidrug resistance phenotype. This phenotype is frequently characterized by a cross-resistance to drugs to which the tumors have not been exposed. The development of a multidrug resistant phenotype in metastatic breast cancer is primarily responsible for the failure of current treatment regimens. The precise nature of this phenotype remains unclear. However, several mechanisms may be involved and include kinetic resistance (*3,4*), gp170 (PGP, P-glycoprotein) expression, glutathione transferases (*5,6*), superoxide dismutases (*7*), topoisomerases (*8*), and the multidrug resistance-associated protein (MRP) (*9*). These may occur independently or in combination, thereby conferring resistance in heavily treated patients exposed to structurally and functionally diverse agents.

In experimental models, the multidrug-resistant phenotype is often accompanied by the expression of the MDR1 gene (also

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See "Notes" following "References."

known as PGY1) and/or its gp170 glycoprotein product (MDR1/gp170, where MDR1 refers to the gene and its messenger RNA and gp170 denotes the glycoprotein gene product) (*10*). A 170-kd cell membrane glycoprotein, gp170 appears to work by actively effluxing substrates from cells (*11*). The glycoprotein possesses two adenosine triphosphate (ATP)-binding sites and exhibits significant ATPase activity (*12*). The drug-binding sites are close to gp170's cytosolic transmembrane domains (*13*), the drugs apparently being removed from within the membrane bilipid layer (*14*). Activation of gp170 may be regulated through phosphorylation by protein kinases, e.g., protein kinase C (*15-17*), and its efflux function may be affected by membrane lipid composition (*18*) and fluidity (*19-21*).

The multidrug-resistant phenotype conferred by gp170 is characterized by resistance to various structurally unrelated anticancer agents, including the anthracyclines, epipodophyllotoxins, vinca alkaloids, and taxanes (*22*). The majority of the most widely used combination chemotherapy regimens for breast cancer include gp170 substrates as either first-line or second-line treatments, most frequently in combination with non-gp170 substrates. For example, doxorubicin (Adriamycin) and vinblastine are gp170 substrates and are among the most effective antineoplastic drugs in breast cancer (*22*). Paclitaxel (Taxol) when administered as a single agent (*23*) or in combination with doxorubicin also produces significant responses in breast cancer (*24*) and is likely to become more widely used as an agent for breast cancer treatment.

The role of MDR1/gp170 in human breast cancer remains unclear, partly because of an apparent lack of a consensus on whether MDR1/gp170 is expressed in breast tumors. While various small studies (*25-27*) have readily detected MDR1/gp170 expression, one of the largest single studies published to date (*28*) and several smaller studies (*29,30*) have failed to detect any expression of MDR1/gp170 in breast tumors. Even among those studies detecting MDR1/gp170 expression, there has been no clear consensus regarding its likely functional significance.

Our primary goals were to clarify the prevalence and clinical relevance of MDR1/gp170 expression in breast cancer and to explore the causes of heterogeneity across the studies evaluated. We have undertaken a critical review and meta-analysis of the published literature describing the expression and potential function of the MDR1 gene and its gp170 product in breast cancer. Meta-analysis has rarely been applied to basic science research. However, it may be useful for translational studies of molecular mechanisms in cancer, since the rapid pace of basic research is associated with considerable variation in methods, reagents, and quality-control measures, and such variation may obscure the clinical relevance of a given molecule.

The specific questions that we addressed are as follows: 1) Is MDR1/gp170 expressed in breast tumors and, if so, what is the frequency of expression? 2) Is MDR1/gp170 expression associated with cytotoxic and/or hormonal treatment and, if so, what is the magnitude of this association? 3) What is the potential functional relevance of MDR1/gp170 expression in breast cancer with respect to response to chemotherapy and predicting prognosis? 4) For each of the above questions, if there is a significant lack of agreement among studies, what factors contribute to this heterogeneity?

Both the validity and the most appropriate methodology for performing reviews of this type are controversial (*31-34*). We have emphasized objective quantitative criteria in our evaluation and have followed explicit guidelines for the conduct of meta-analyses and critical research reviews (*35-37*). Moreover, we considered an assessment of the sources of heterogeneity to be a goal of the analysis rather than a preliminary step.

The treatment and exposures of interest are the systemic therapies administered to breast cancer patients. Such therapies include cytotoxic chemotherapy, which we considered in two subsets, i.e., regimens including at least one known gp170 substrate and regimens not including any known substrates. For example, the CAF regimen (cyclophosphamide, doxorubicin, and fluorouracil) is considered to be an MDR1/gp170-related drug regimen because of the presence of doxorubicin. In contrast, the CMF regimen (cyclophosphamide, methotrexate, and fluorouracil) is considered to be a non-MDR1/gp170-related drug regimen. We also have included studies that utilized the antiestrogen tamoxifen (*38,39*), which is known to reverse gp170-mediated resistance in vitro.

Materials and Methods

Criteria for Conducting the Review and Meta-analysis

Formal research reviews and meta-analyses have become increasingly common for evaluating large, often diverse bodies of research to resolve apparent lack of agreement among the individual studies. These often take the form of purely synthetic exercises, where the individual study results are combined in a summary measure, without regard to the appropriateness of combining the studies. Such analyses often also lack a careful exploration of the reasons for disagreement among studies. The latter can often be as important as or more important than the summary measure, since the factors underlying disagreement may point to informative patient subgroups or methodologic issues.

This review and meta-analysis focused not only on quantifying the prevalence of MDR1/gp170 expression and its functional relevance in breast cancer, but also on exploring sources of variation or heterogeneity in results across studies. The appropriate criteria for research reviews and meta-analyses have been widely discussed (*37,40-42*), most recently in this Journal (*43*). The general guidelines from these sources were adapted and applied for the conduct of this review as follows:

1) We include a clear statement of the specific purpose(s) of the review, with reference to the population to be generalized, the treatment or exposure of interest, and the major outcome(s) of interest (*see above*).
2) We describe the sources and define the methods of citation searches.
3) We formulated specific guidelines, in advance, to determine which studies were to be excluded or included and detail the reasons for exclusion or inclusion.
4) We established, and subsequently applied, a consensual assessment of the validity of the methods used in the studies reviewed and provide a determination of what conclusions are justified by these methods. More than one author was involved in determining the assessment.
5) We integrate results of individual studies in a quantitative, weighted fashion, with consideration of data limitations and/or inconsistencies. Where significant heterogeneity is present, we attempt to determine its sources. This may be more important than any summary measure.
6) We summarize all major or relevant findings.
7) We identify specific directions or considerations for new research by identifying gaps in our present knowledge.
8) We provide suggestions for the design of future studies, particularly where this relates to eliminating confounding factors and identified sources of heterogeneity.

Sources of Data and Methods of Citation Search

To identify studies that evaluated MDR1/gp170 expression in human breast cancers, we performed an extensive literature search of the following databases: Medline (National Library of Medicine, National Institutes of Health, Bethesda, MD), CANCERLIT (National Library of Medicine), *Current Contents* (Institute for Scientific Information, Philadelphia, PA), *Current Advances in Cancer Research* (Current Awareness in Biological Sciences, Elsevier Science Inc., Tarrytown, NY), *Knowledge Finder* (Aries Systems Corp., North Andover, MA), and the *Science Citation Index* (Institute for Scientific Information). For keyword-based Boolean searches, we used 44 different keywords that were either words or variations of words, e.g., spelling, punctuation, and abbreviation, which represent the multidrug resistance subject. We also used the bibliography listings of relevant papers found in the above databases as leads for identifying additional studies.

Criteria for Inclusion or Exclusion

For studies to be included, they had to describe original research involving measurement of MDR1/gp170 expression in human breast cancers. Reviews were excluded. If more than one report described the same data, we used only the latest report. To be included, a published report also must have enabled determination of the number of MDR1/gp170-positive and -negative tumors. Since amplification is often an unreliable indicator for gene expression and the contribution of other relevant coamplified genes cannot be assessed readily (*44,45*), studies based only on amplification of the MDR1 gene were not included. All other studies, which primarily used immunologic detection of protein (western blot or immunohistochemistry) and/or RNA hybridization methods (northern blot or dot blot), were included. Study eligibility was determined independently by all three investigators. When determining eligibility, the investigators were not formally blinded to study results because it was often necessary to read the results to determine whether the number of MDR1/gp170-positive tumors was given. However, the inclusion criteria are quite unambiguous, and it is unlikely that lack of blinding induced bias (only four papers were excluded—*see below*).

Three studies (*28,46,47*) used more than one assay method to evaluate the same group of tumor samples. These results were included in all analyses of subgroups of studies that used a specific method. For example, the report by Merkel et al. (*28*) analyzed many of the same tumors by use of both western blot and RNA hybridization methods and was represented in both the subgroups of western blot and RNA hybridization studies. (Data from the study by Merkel et al. that were based on gene amplification were not included.) In analyses that pooled *all* studies, the results obtained by Merkel et al. were represented by the assay method based on the largest number of tumors (western blot). The results obtained by Kim (*46*), who included the same number of tumors for each assay method, were represented by the method yielding the highest expression of MDR1/gp170, i.e., immunohistochemistry. The study by Chevillard et al. (*47*) is represented here among the studies of immunohistochemistry. There were insufficient studies using the polymerase chain reaction (PCR) to enable analysis of data obtained by this methodology.

Assessment of Studies and Methods

Studies of MDR1/gp170 expression in breast cancer are essentially translational research. Thus, methodologic considerations must be coupled with the ability to derive clinical inferences from the results. With this in mind, we chose to assess the methodologic rigor and the clinical relevance of the studies by applying a series of objective technical criteria. These criteria were used to describe the studies, but a "quality score" was not obtained for use as a stratification factor for the meta-analysis. Such quality scores are often confounded and highly subjective. Stratification or exploration of heterogeneity can be better accomplished on the basis of the individual components of the quality score (*33*).

The criteria that we used were based on factors that we deemed a priori to be potential contributors to heterogeneity and that also comprised a set of important criteria for the conduct of this type of translational research. The criteria included the following:

1) Appropriate consideration of statistical precision, i.e., adequate sample size, use of confidence intervals (CIs), or consideration of sample size in interpretation of results. Twenty-five patients were considered to be the minimum adequate sample size because this size would produce an upper confidence bound of less than 10% if the study found no tumors expressing MDR1/gp170.
2) Adequate description of tumors and treatment, such that MDR1/gp170-positive and -negative tumors could be classified according to whether the assay was performed on primary or metastatic tumors (before or after chemotherapy) and according to whether treatment included multidrug-resistant substrates (e.g., doxorubicin) or non-multidrug-resistant substrates (e.g., cyclophosphamide).
3) A clear description of the criteria for MDR1/gp170 positivity, sufficient to allow comparison among studies.
4) A clear description of the characteristics of the patient population and criteria for accrual.
5) The presence of positive controls in the assay for MDR1/gp170 expression.
6) The presence of negative controls in the assay for MDR1/gp170 expression.
7) The use of more than one antibody for assessing gp170 expression (immunohistochemistry studies only) and the use of non-cross-reacting antibodies or confirmatory methods such as PCR.

These criteria were established in advance by all three authors, who also scored each study independently. Each criterion was scored simply as (+) or (−) by each assessor.

Statistical Methods, Integration of Individual Studies, and Assessment of Heterogeneity

The proportion of tumors expressing MDR1/gp170 was calculated in each study and in subgroups where appropriate. A 95% CI for the proportion was calculated by use of standard methods for the binomial distribution. The proportions were pooled across studies as the weighted average of the proportions with the use of individual study sample sizes as weights (*48*). A chi-squared test for homogeneity (χ^2_{homog}) of the proportions was calculated by use of standard methods for 2 × C contingency tables (*49*). It should be noted, however, that the test for heterogeneity may be too sensitive when the number of studies (number of columns in the contingency table) is large.

To explore sources of significant heterogeneity among the studies, we examined whether the proportion of MDR1/gp170-expressing tumors exhibited a linear trend with any characteristics of the individual studies. We applied a weighted regression model, with the study-specific proportion as the dependent variable and weighted by the sample size. This analysis was performed by use of the weighted regression option in the procedure PROC REG in SAS (Statistical Analysis Systems Institute Inc., Cary, NC). Since each "observation" was an average over all patients in a study, the standard errors of the regression coefficients were corrected by dividing by the square root of the residual mean square (*50*).

We also examined heterogeneity by classifying studies according to potential sources of heterogeneity and recalculating the weighted mean proportions and χ^2_{homog} within these subgroups. To compare subgroups of studies, e.g., studies in which MDR1/gp170 was measured before versus after chemotherapy, we calculated the weighted mean of the proportion expressing MDR1/gp170 for each subgroup of studies. The difference between means was divided by the standard deviation of the difference to give a *z*-score as described by Greenland (*50*).

We examined the potential association between MDR1/gp170 expression and 1) prognostic factors, 2) prior treatment, 3) clinical response to chemotherapy, and 4) in vitro doxorubicin resistance. We determined the relative risk (RR) of the particular clinical outcome in MDR1/gp170-positive versus -negative tumors for each study and pooled the RRs across studies by using a Mantel–Haenszel approach (*51*). Cochran–Mantel–Haenszel statistics (*52*), as implemented in the SAS subroutine PROC FREQ, were used to test the significance of the pooled RR. A similar approach was used to investigate the effect of chemotherapy on MDR1/gp170 induction, but the RR compared the probability of MDR1/gp170 induction between patients with and without prior chemotherapy at the time of MDR1/gp170 measurement. CIs for the pooled RRs and a χ^2_{homog} of RRs were calculated by use of the method of Breslow and Day (*51*). These analyses were conducted by the procedure PROC FREQ in SAS. Since many studies had small cell sizes or cells with zero patients, we included the continuity correction of 0.5 added to each cell for the calculation of RRs. We used pooled RRs rather than odds ratios because the latter can overestimate the RR when the outcome being considered occurs in more than 10% of the study sample (*53*). Since the samples in these studies were sampled in a cohort fashion, there is no need to use the odds ratio because the RR provides a clearer indication of the clinical impact of MDR1/gp170 expression. All *P* values are two-sided.

Results

Results of Citation Search

Thirty-one studies (*25-30,46,47,54-76*) were identified that met the criteria for inclusion in the meta-analysis. These included 21 studies (*26,46,47,55,57-59,61,62,64-75*) with MDR1/gp170 expression based on immunohistochemistry, eight studies (*27-29,46,54,56,60,63*) based on RNA hybridization methods, three studies (*25,28,30*) based on immunoblot (western blot) methods, and two studies (*47,76*) based on the reverse transcription–PCR. [Three studies (*28,46,47*) used more than one method.] One additional study (*77*) was excluded from the analysis because it used only gene amplification as an indicator of MDR1/gp170 activity. Twelve studies (*30,46,54,56,59,61,64-66,70,72,75*) measured MDR1/gp170 expression on tumors prior to any chemotherapy or hormonal therapy, 13 studies (*25-28,47,55,57,62,63,67,73,74,76*) included tumor measurements both before and after treatment, two studies (*68,69*) had only post-treatment tumor measurements, and in four studies (*29,58,60,71*) the authors did not indicate the timing of the assay with respect to treatment. In addition to the studies that met the inclusion criteria, four published reports identified by the search were excluded for the following reasons: Only gene amplification was used as an indicator of MDR1 activity (*77*), results for breast tumors could not be separated from those for all tumors (*78*), no samples from breast cancers were tested (*79*), and the report described earlier results that are included in a later report (*80*).

Assessment of Methodologic Rigor of Studies

Table 1 lists the degree to which the individual studies complied with the criteria for methodologic rigor. Only two of the criteria, i.e., a definition of MDR1/gp170 positivity and the use of positive and negative controls, were met by nearly all studies. For each of the other four criteria, there were many studies that did not adhere to at least one criterion. Thirty-five percent of the studies had a sample size of fewer than 25 patients. None of these 11 studies (*26,29,30,46,54,55,58,59,61,74,76*) used CIs to indicate the degree of uncertainty in their data, and they did not discuss the possible influence of sample size on their results. Because small samples produce relatively imprecise estimates of the prevalence of MDR1/gp170 expression and its association with clinical parameters, sample size may contribute to hetero-

Table 1. Methodologic and clinical criteria for studies of MDR1/gp170 expression in human breast cancer*

Authors, year of publication (reference No.)	Criteria								
	Sample size	Description of tumor, treatment†	Definition of MDR1/gp170 positivity‡	Description of patient base§		Controls§		Adequacy of antibody methods‖	
						Positive	Negative		
Goldstein et al., 1989 (*27*)	+	−	+	−	−	+	+	NA	NA
Kacinski et al., 1989 (*54*)	−	+	+	−	+	+	+	NA	+
Merkel et al., 1989 (*28*)	+	+	+	−	−	+	−	−	(+)
Moscow et al., 1989 (*29*)	−	−	+	−	−	+	+	NA	NA
Ronchi et al., 1989 (*30*)	−	+	+	−	−	+	+	−	−
Schneider et al., 1989 (*55*)	−	+	+	−	−	+	+	−	−
Salmon et al., 1989 (*26*)	−	−	+	−	−	+	+	+	+
Keith et al., 1990 (*56*)	+	+	+	−	−	+	+	NA	NA
Ro et al., 1990 (*57*)	+	+	+	+	+	+	+	−	−
Wishart et al., 1990 (*66*)	+	+	+	−	−	+	+	+	+
Sugawara, 1990 (*59*)	−	−	−	−	−	+	+	−	−
Kim, 1990 (*46*)	−	+	+	−	−	+	+	−	+
Cordon-Cardo et al., 1990 (*58*)	−	−	−	−	−	+	+	+	+
Wallner et al., 1991 (*60*)	+	−	+	−	+	+	+	NA	NA
Verrelle et al., 1991 (*61*)	−	+	+	−	+	+	+	−	−
Sanfilippo et al., 1991 (*25*)	+	−	+	−	−	+	+	−	−
Dixon et al., 1992 (*68*)	+	−	+	−	−	+	+	−	−
Koh et al., 1992 (*62*)	+	+	+	−	−	+	+	−	−
Hennequin et al., 1993 (*63*)	+	+	+	−	+	+	+	NA	NA
Botti et al., 1993 (*69*)	+	+	+	−	+	+	+	−	−
Schneider et al., 1994 (*9*)	+	+	−	−	+	−	+	+	+
Veneroni et al., 1994 (*64*)	+	+	+	−	−	+	+	−	−
Charpin et al., 1994 (*65*)	+	+	+	−	+	+	+	−	(+)
Keen et al., 1994 (*67*)	+	−	+	−	−	−	−	−	−
Schneider and Romero, 1995 (*75*)	+	+	+	+	+	+	+	−	−
Seymour et al., 1995 (*73*)	+	+	+	−	+	+	+	−	−
Linn et al., 1995 (*71*)	+	−	+	−	+	+	+	+	+
Decker et al., 1995 (*74*)	−	+	+	+	+	+	+	+	+
Luowen et al., 1995 (*72*)	+	+	+	+	+	+	+	−	−
Chevillard et al., 1996 (*47*)	+	+	+	−	+	+	+	+	+
O'Driscoll et al., 1996 (*76*)	−	+	−	−	−	+	+	NA	NA

*+ = criterion present; − = criterion absent; (+) = criterion partially achieved; NA = not applicable.

†This criterion addresses whether MDR1/gp170 expression could be classified as to primary versus metastatic tumor, treated versus untreated patients, and treatments that are or are not g170 substrates.

‡This criterion addresses whether the description is sufficient to allow comparison of MDR1/gp170 positivity among studies.

§The first rating concerns the description of how samples were accrued; i.e., is it sufficient to assess the likely sources of bias? The second rating concerns the description of characteristics of the study population; i.e., can individual characteristics be related to multidrug resistance?

‖Refers to whether studies based on antibody methods were likely to have guarded against artifacts by (first column) use of more than one antibody (indicated by +) or (second column) by use of non-cross-reacting antibodies or other confirmatory methods (indicated by +).

geneity. Thirty-two percent of the studies did not provide a sufficient description to determine whether tumor samples came from primary or metastatic tumors or from treated or untreated patients and the type of treatment(s) used, or they did not use treatments known to be gp170 substrates. As a result, it is difficult to interpret the observed prevalence of MDR1/gp170 expression. Most studies did not adequately describe the patient base or accrual procedure. This information is important for determining the representativeness of patients in an individual study and the ability to generalize study results. It also allows the reader to determine a study's susceptibility to various forms of bias (e.g., size bias, defined as bias toward larger tumors that allow multiple sampling).

Fourteen studies (*46,55,57,59,61,62,64,65,67-69,72,73,75*) of the 21 (67%) studies based on immunohistochemical detection of gp170 and all three studies (*25,28,30*) using the western blot technique used only one antibody (Table 1), despite the commercial availability of several antibodies with nonoverlapping epitopes (*81-84*). Unfortunately, several of the antibodies used in isolation have well-defined cross-reactivity with proteins other than gp170. For example, both JSB-1 (*85*) and C494 (*86*) cross-react with pyruvate carboxylase. The C219 monoclonal antibody recognizes MDR3, a member of the same gene family that is not involved in drug resistance (*87*). This antibody also cross-reacts with the heavy chain of muscle myosin (*84*), and myofibroblasts are a major component of the desmoplastic response to breast cancer (*88*). Thus, there may be a tendency for an increased frequency of false-positive results in some single-antibody studies, where the adequacy of the negative controls becomes critical for assessing the likely validity of gp170 estimates.

Proportion of MDR1/gp170-Positive Tumors

Panels A and B of Fig. 1 show the proportion of MDR1/gp170-positive tumors and the associated 95% CIs for studies with MDR1/gp170 measured before or after treatment, respectively. Expression varies considerably across studies; this variation is apparent regardless of whether the tumor material was assayed before or after chemotherapy. Some potential sources of heterogeneity are indicated by stratifying the studies according to the type of assay method (Fig. 1, A) or the type of treatment received (Fig. 1, B). Fig. 1, A, shows that most of the studies that found no expression of MDR1/gp170 were based on western blot or RNA hybridization methods. Only one immunohisto-

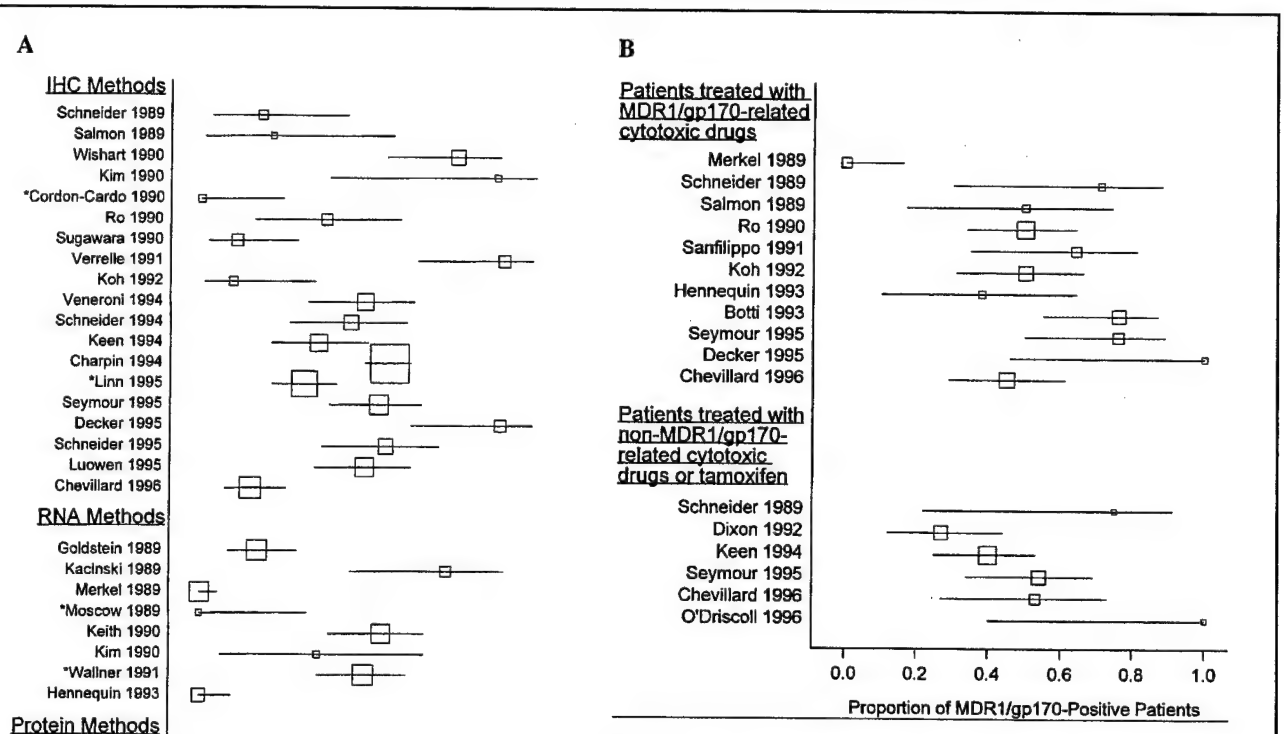

Fig. 1. A) MDR1/gp170 expression measured in breast cancer before treatment. Data represent the proportion of MDR1/gp170-positive tumors and the associated 95% confidence interval. Studies are grouped according to the method used for MDR1/gp170 detection. IHC = immunohistochemistry; RT-PCR = reverse transcription–polymerase chain reaction. * = tumors in these studies were assumed to be from previously untreated patients, but the description in the published reports did not allow this to be determined with certainty. **B)** MDR1/gp170 expression measured in breast cancer after cytotoxic or hormonal therapy. Data represent the proportion of MDR1/gp170-positive tumors and the associated 95% confidence interval. Studies are grouped according to whether patients have been treated or not treated with anticancer drugs that are gp170 substrates.

chemistry study (*58*) found no immunoreactivity (based on only eight breast tumors), but this study reported some staining of "apparently normal parenchymal" cells in some specimens.

Based on the weighted average over all 31 studies listed in Table 1, the percentage of tumors expressing MDR1/gp170 was 41.2% (95% CI = 36.0%-46.5%). However, the proportions were highly variable across individual studies ($P<.0001$). These studies were performed over an 8-year period (1989-1996). To evaluate whether potential sources of heterogeneity may have changed over time, we performed a weighted linear regression of the proportion of MDR1/gp170-positive tumors in each study against the calendar year when the study was published. Table 2 shows a highly significant trend with time, there being a slope coefficient of 0.040, with $z = 3.52$ ($P = .0005$). This trend indicates that, on average, there was an increase of about 4.0% per year in the reported detection of MDR1/gp170 expression in breast tumors.

There was a shift away from RNA hybridization methods toward immunohistochemical methods over this time, so we stratified the studies according to whether they used RNA hybridization or immunohistochemical methods and conducted separate regressions within each group of studies. (No regression was conducted for the western blot studies because there were only three such studies.) Within groups of studies using either RNA hybridization or immunohistochemical methods, no trend of MDR1/gp170 expression with time was evident (Table 2). Thus, a significant degree of variability among the studies appears to be due to the shift over time from RNA hybridization methods (primarily northern blot hybridizations) to the more sensitive immunohistochemical methodologies.

We next examined heterogeneity separately within both the RNA hybridization and immunohistochemistry studies. The average percentage of tumors expressing MDR1/gp170 was 27.1% (95% CI = 15.4%-38.6%) for studies using RNA hybridization methods and 48.5% (95% CI = 42.0%-55.0%) for immunohistochemistry studies. However, even within these subgroups, there was still significant heterogeneity ($P<.0001$ for both RNA hybridization and immunohistochemistry studies).

We further stratified these subgroups according to whether MDR1/gp170 had been measured before or after chemotherapy or hormonal therapy. The difference between values before and after chemotherapy or hormonal therapy was relatively small for both immunohistochemistry and RNA hybridization studies, and significant heterogeneity still existed within these subgroups (Table 3). Among studies of values after chemotherapy or hormonal therapy, we compared MDR1/gp170 expression among tumors from patients treated with and without drugs associated with the multidrug-resistant phenotype (data not shown). As expected, MDR1/gp170 expression was higher for patients treated with MDR1/gp170-related drugs, with an average of 57.2% of tumors being positive. However, a sizable percentage (44.6%) of patients treated with non-MDR1/gp170-related drugs also expressed MDR1/gp170. The studies of MDR1/gp170-related and non-MDR1/gp170-related drugs exhibited marginally significant heterogeneity ($P = .052$ and $P = .054$, respectively).

Table 2. Regression of proportion of tumors expressing MDR1/gp170 on year in which the study was conducted (expressed as year − 1900)

Studies	Regression coefficient	Corrected standard error	*z*-statistic	*P**
All studies† (n = 31)	0.040	0.011	3.52	.0005
Immunohistochemistry studies (n = 21)	0.004	0.017	0.23	.82
RNA hybridization studies (n = 8)	0.019	0.044	0.43	.67

*Two-sided.

†Includes two studies based only on western blot methods (*25,30*) and one study that used both western blot and RNA hybridization methods (*28*).

Associations With Clinical Parameters

It has been suggested that the expression of MDR1/gp170 is more likely to be a surrogate marker for a worse prognosis than an indicator of potential response to cytotoxic chemotherapy (*89*). To address the clinical relevance of MDR1/gp170 expression, we considered the evidence linking it to various clinical parameters, including induction by cytotoxic or hormonal treatment and association with 1) established prognostic attributes, 2) response to chemotherapy, 3) recurrence and survival, and 4) in vitro doxorubicin resistance.

Induction of MDR1/gp170 expression. The effect of chemotherapy on induction of MDR1/gp170 expression is shown in Table 4. This analysis was performed by comparing the proportion of patients whose tumors expressed MDR1/gp170 before chemotherapy with that of patients whose tumors expressed MDR1/gp170 after chemotherapy. We used only studies that provided both pretreatment and post-treatment data, although most were not consecutive measurements on the same patients. In the 13 studies meeting this criterion (*25-28,47,55,57,62,63,67,73,74,76*), treatment with cytotoxic or hormonal agents was associated with a significant increase in the proportion of tumors expressing MDR1/gp170 (RR = 1.77 [95% CI = 1.46-2.15]; $P<.0001$). Unlike the analysis performed on all stud-

Table 3. Pooled proportion of tumors expressing MDR1/gp170, according to timing of assay with respect to treatment (pretreatment versus post-treatment), and assay method (immunohistochemistry based versus RNA hybridization based)

Timing (No. of studies)	Assay	No. of patients	Pooled proportion	Test of homogeneity	
				χ^2 (df*)	*P*†
Pretreatment (17)	Immunohistochemistry	661	0.458	91.6 (16)	<.001
Pretreatment (8)	RNA hybridization	270	0.274	72.8 (7)	<.0001
Post-treatment (10)	Immunohistochemistry	279	0.520	23.8 (9)	.005
Post-treatment (3)	RNA hybridization	22	0.227	10.6 (2)	.005

*df = degrees of freedom for the test of homogeneity.

†Two-sided.

Table 4. MDR1/gp170 expression in association with cytotoxic therapy*

Type of treatment	No. of studies (No. of patients)	Summary: RR (95% CI)	RR P value†	Test for homogeneity χ^2 (df)	Test for homogeneity P†
All cytotoxic drugs	13 (726)	1.77 (1.46-2.15)	<.0001	20.22 (12)	.056
MDR1/gp170-related drugs only	10 (499)	1.99 (1.56-2.54)	<.0001	12.81 (9)	.19

*RR = relative risk, i.e., the probability of MDR1/gp170-positive tumor in patients treated with cytotoxic therapy versus untreated patients; CI = confidence interval; df = degrees of freedom for the test of homogeneity.
†Two-sided.

ies, there was only marginally significant heterogeneity in this subgroup (P = .056). When we excluded the three studies that used drugs not commonly related to MDR1/gp170 (*27,67,76*), the effect was somewhat stronger (RR = 1.99 [95% CI = 1.56-2.54]; P<.0001), and there was no significant heterogeneity (P = .19). The results were unchanged when we repeated the analyses by reclassifying as negative those tumors exhibiting weakly positive expression (*25,55,57*).

Association with prognostic attributes. Only 11 studies (*54,57,60,61,63,65,69,70,73-75*) included data associating MDR1/gp170 expression with one or more known prognostic attributes, including lymph node status at diagnosis, tumor size, tumor histology, tumor grade, and estrogen receptor status. We considered only attributes examined in three or more studies. Table 5 shows that none of these attributes were significantly associated with MDR1/gp170 expression. This result suggests that MDR1/gp170 expression is not acting as a surrogate for another prognostic factor in its ability to predict outcome or response to chemotherapy.

Association with response to chemotherapy. Since studies provided different levels of detail about clinical response, we considered all studies in which it was possible to determine the number of patients who exhibited at least a clinical partial response, i.e., either partial response (PR) or complete response (CR). These responses were defined according to widely accepted usage, where CR indicates complete disappearance of the tumor, while PR indicates a greater than 50% reduction in the largest diameter of the tumor. There were nine studies in which these data could be determined (*47,57,61-64,68,69,74*). An additional three studies either did not permit classification of the responses of patients into CR/PR versus less than PR (*46,67*) or did not permit the association between MDR1/gp170 positivity versus negativity (*73*).

Table 6 shows that patients whose tumors expressed MDR1/gp170 were 3.21 times more likely to exhibit a worse than PR than patients whose tumors did not express MDR1/gp170 (P<.0001). There was no evidence of significant heterogeneity among these studies (P = .27). Excluding the single study using treatment with non-MDR1/gp170-related drugs did not significantly change the association. Furthermore, the association between MDR1/gp170 expression and clinical response became stronger when the studies were restricted to those in which expression was measured after any treatment (RR = 4.19 [95% CI = 2.71-6.47]; P<.0001) or after treatment with MDR1/gp170-related drugs (RR = 3.87 [95% CI = 2.44-6.14]; P<.0001) (Table 6). This is consistent with the ability of MDR1/gp170-related drugs to induce MDR1/gp170 expression and the ability of this induced expression to confer cross-resistance among classical substrates for gp170 but not for other drugs (*10,90-92*). When we repeated the analyses by reclassifying as negative those tumors exhibiting weak positive expression (*61,69,74*), the results were unchanged.

An important clinical concern is whether the expression of MDR1/gp170 before initial chemotherapy will predict response. Only five studies (*47,57,61,64,74*), with a total of 115 patients, measured expression before treatment in patients evaluated for response to subsequent chemotherapy. When combined (χ^2_{homog} = 1.98; P = .74), these studies indicate a suggestive, but not statistically significant, association between MDR1/gp170 expression prior to treatment and a worse than partial clinical response to cytotoxic regimens containing gp170 substrates (RR = 1.47 [95% CI = 0.94-2.29]; χ^2_{homog} = 2.91; P = .088).

Association with recurrence and survival. Eight studies (*61,63,69,71-75*) associated MDR1/gp170 expression with either recurrence-free survival or overall survival. Unfortunately, conducting any formal statistical evaluation of the combined studies was not possible because the necessary data were included in only two studies that evaluated recurrence-free survival (*63,69*) and in three studies that evaluated overall survival

Table 5. Associations between MDR1/gp170 expression and breast cancer prognostic factors*

Prognostic factor	No. of studies (No. of patients)	Summary: RR (95% CI)	RR P value†	Test for homogeneity χ^2 (df)	Test for homogeneity P†
Positive lymph nodes: yes versus no	9 (255)	1.08 (0.89-1.32)	.43	0.52 (8)	.99
Estrogen receptor status: negative versus positive	5 (426)	1.08 (0.84-1.39)	.57	4.56 (4)	.35
Tumor size: T3/T4 versus T1/T2	7 (184)	0.92 (0.75-1.13)	.44	1.63 (6)	.98
Histology: ductal versus lobular	5 (176)	0.96 (0.86-1.06)	.42	0.04 (4)	.99
Tumor grade: grade 3 versus grade <3	3 (58)	1.74 (0.65-4.64)	.27	1.56 (2)	.47

*RR = relative risk, i.e., the risk of adverse prognostic factor category among those patients with MDR1/gp170-positive versus MDR1/gp170-negative tumors; CI = confidence interval; df = degrees of freedom for the test of homogeneity. *See* (*120,121*) for staging and grading systems used for the tumors.
†Two-sided.

Table 6. Association between MDR1/gp170 expression and failure to respond (i.e., less than a clinical partial response) to cytotoxic drugs*

Type of treatment	No. of studies (No. of patients)	Summary: RR (95% CI)	RR P value†	Test for homogeneity	
				χ^2 (df)	P†
All cytotoxic agents, pretreatment or post-treatment MDR1/gp170 expression measurements	9 (260)	3.21 (2.28-4.51)	<.0001	10.0 (8)	.27
MDR1/gp170-related drugs, pretreatment or post-treatment MDR1/gp170 expression measurements	8 (215)	2.97 (2.08-4.25)	<.0001	9.18 (7)	.24
All cytotoxic agents, post-treatment MDR1/gp170 expression measurement	7 (193)	4.19 (2.71-6.47)	<.0001	7.64 (6)	.27
MDR1/gp170-related drugs, post-treatment MDR1/gp170 expression measurement	6 (135)	3.87 (2.44-6.14)	<.0001	7.41 (5)	.21

*RR = relative risk, i.e., the risk of less than a clinical partial response to chemotherapy among those patients with MDR1/gp170-positive versus MDR1/gp170-negative tumors; CI = confidence interval; df = degrees of freedom for the test of homogeneity.
†Two-sided.

(*63,74,75*). However, of the four studies that associated MDR1/gp170 expression with recurrence-free survival, two (*61,69*) found reduced survival associated with expression, whereas the other two (*63,71*) did not. Similarly, three studies (*71,72,75*) found that expression was associated with a significant reduction in overall survival, whereas three other studies (*63,73,74*) did not. Only two studies (*71,72*) used multivariable analysis to control for other major prognostic factors, although both studies observed significant survival decrements even in univariate analyses.

Association with in vitro doxorubicin resistance. Three studies (*25,26,64*), with a total of 93 patients, examined the association between MDR1/gp170 expression and resistance to doxorubicin using an in vitro clonogenic assay on cells from patients' tumors. Patients whose tumors expressed MDR1/gp170 were 2.5 times more likely to exhibit in vitro resistance to doxorubicin (RR = 2.50; 95% CI = 1.77-3.52) (P<.0001). There was no evidence of significant heterogeneity in this group of studies (χ^2_{homog} = 4.15 [2 degrees of freedom]; P = .14). These studies indicate that some breast tumor cell subpopulations express sufficient levels of MDR1/gp170 to confer a functional level of in vitro drug resistance.

Discussion

The considerable variability across studies of MDR1/gp170 expression explains the controversy regarding the presence of this expression and its relevance in breast cancer. A cursory evaluation of this literature could lead to apparently equally justifiable support for quite divergent opinions. For example, it could be argued and supported by several citations either that MDR1/gp170 expression is rarely detected in breast cancer and has no clinical relevance or that expression is widely detected and may have considerable clinical relevance. With such variability across many independent studies, a careful review and meta-analysis can provide a critical and objective approach to the body of research.

Purpose and Interpretation of Meta-analyses

In its simplest form, meta-analysis is a systematic approach to combining, in a quantitative fashion, the results of related studies. The approach and its justification are conceptually similar to those used in either multicenter studies or studies with a large number of potentially relevant subgroups or strata. Combining the results of individual studies is performed in a manner similar to that of combining the results across strata in a single large study. However, the validity of such data pooling and the generation of a summary statistic require that the studies be sufficiently alike in the association being measured. Thus, both the study population and methods should be sufficiently similar that it is reasonable to consider all the studies as if they had been generated as subgroups of a single large study. This is rarely the case, or there would be little controversy with regard to the overall inference from the studies and there would be little need to conduct a meta-analysis (*33*).

As occurred with the MDR1/gp170 analyses, groups of studies typically exhibit meaningful variability, and the search for the source of the variability is important. This search may also identify subgroups of studies that are sufficiently alike to make data pooling appropriate. For example, this meta-analysis showed that significant heterogeneity in the proportion of tumors expressing MDR1/gp170 could be attributed to differences in types of assays (immunohistochemistry based versus RNA hybridization based), complicating meaningful interpretation of a summary measure of this proportion.

Where subgroups of studies are chosen on a rational and predefined basis and the data from the integrated studies do not exhibit significant heterogeneity, meaningful associations among variables can be identified. For example, all nine of the studies that contain information on MDR1/gp170 expression and response to chemotherapy (*47,57,61-64,68,69,73*) can be validly combined and explored because there is no significant heterogeneity (Table 6; χ^2_{homog} = 10.0; P = .27).

One concern in the conduct of any meta-analysis is publication bias, i.e., the tendency for studies reporting "negative" results to be underrepresented in the literature. Fig. 2 shows a "funnel plot," where the proportion of MDR1/gp170-positive tumors in each study is plotted against the sample size (as an indicator of standard error). In the absence of publication bias, such a plot should resemble a funnel lying on its side with the narrow end pointing to the right, with study results scattered around an expected "true" value and the degree of scatter decreasing with sample size. Publication bias would be suggested by a lack of small "negative" studies, i.e., studies with zero or low percentages of tumors expressing MDR1/gp170 (*93*). Fig. 2 shows no apparent bias against negative studies, since six studies (*28-30,58,59,63*) demonstrated expression in 10% or less of tumors, and four of these studies (*29,30,58,59*) had 20 patients or fewer.

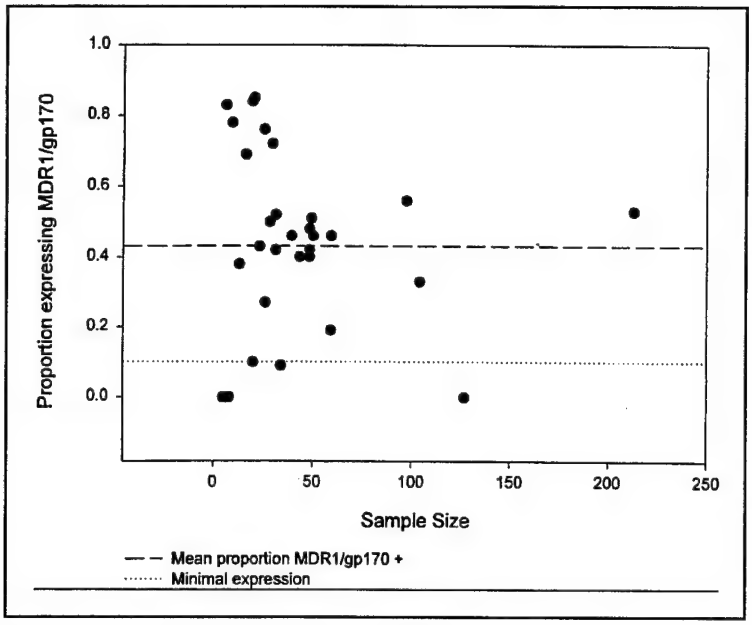

Fig. 2. Funnel plot evaluation of publication bias in studies of MDR1/gp170 expression and breast cancer. Data represent the proportion of MDR1/gp170-positive tumors (from untreated plus treated patients) in each study, plotted against the study sample size.

Frequent but Heterogeneous Expression of MDR1/gp170 in Breast Cancer

Northern and western blot hybridization techniques suffer from the possible loss of the signal by dilution from surrounding MDR1/gp170-negative tissues. This result may account for the generally lower estimates of positivity in the studies using these techniques when compared with immunohistochemistry. For many years, the single largest study in breast cancer provided the most compelling evidence for a lack of expression and, therefore, of any functional role of MDR1/gp170 in breast cancer (*28*). Merkel et al. (*28*) acknowledge that their analysis, which utilized northern, Southern, and western blotting analyses of homogenates of breast tumor tissues, may be too insensitive to adequately quantitate MDR1/gp170 expression. This may be the most likely explanation for the lack of detectable MDR1/gp170 expression in breast tumors in their study, since expression has been readily detected in more recent studies of comparable size (*65,71,73*).

As the results of our analysis indicate, the increase over time in the use of more sensitive immunohistochemistry-based assays has been accompanied by an increase in the reported prevalence of MDR1/gp170 expression. An inability to detect gene expression by use of earlier RNA hybridization or immunoblot assay techniques is not surprising, given the generally heterogeneous and low levels of expression now apparent in many breast tumors (*62,66,73*). Immunoblotting has recently been recommended as one means to detect MDR1/gp170 expression in tumors (*94*). However, it is apparent from our study that this approach is inappropriate for analysis of the relatively low levels of MDR1/gp170 expression in breast tumors.

While one source of heterogeneity arises from the use of different assays for MDR1/gp170 expression, significant heterogeneity remains when studies using similar assay techniques are combined. This applies to both RNA hybridization and immunohistochemical analyses. There are clearly many other sources of heterogeneity (e.g., differences in patient populations resulting from patient selection criteria; whether there has been prior treatment, the nature of the treatment, and whether it includes gp170 substrates; the timing of sampling relative to diagnosis and treatment; the use of primary versus metastatic tumor tissue; and sampling artifacts reflecting heterogeneous expression within the tissue). Additional sources of heterogeneity specific to different types of assays include tissue handling and preparation, the choice of antibody(ies) or probes, general laboratory procedures for the particular assay, and the choice of an immunodetection method. Differences in the criteria for scoring tumors as positive or negative also contribute significantly to heterogeneity. Several workshops and individual laboratories (*94-98*) have described their protocols for optimal detection of MDR1/gp170. With such diversity of opinion and laboratory practice, obtaining an internationally recognized standardized approach may be difficult in the short term.

The significant heterogeneity across studies prevents the generation of a statistically valid summary estimate to describe the overall prevalence of MDR1/gp170 expression in breast cancer. However, the weighted average over all 31 studies provides a very general approximation and implies that approximately 40% of all breast tumors express detectable levels of MDR1/gp170. This may be an underestimate, since it combines the typically lower positivity rates found in the less sensitive RNA hybridization studies. While less than the frequency of positivity for estrogen receptor expression, which is almost 70% in all breast tumors when assayed by immunohistochemistry (*99-101*), the frequency of gp170 positivity exceeds that of other prognostic indicators. For example, overexpression of erbB2 when detected by immunohistochemistry approaches 20% in all breast cancers and 25% in invasive ductal cell carcinomas (*102*). This overexpression also may be associated with reduced sensitivity to some chemotherapeutic regimens (*103*). p53 mutations are considered the most frequent genetic changes in breast cancer; they are found in approximately 15%-50% of breast cancers (*104,105*), a frequency that compares with our estimate of 40% MDR1/gp170 positivity. With such a high relative frequency for detection of MDR1/gp170, a definitive assessment of its functional role in breast cancer is an important goal.

MDR1/gp170 Expression in Untreated and Treated Breast Cancers

Many previously untreated breast cancers, 27% in the RNA hybridization studies and 46% in the immunohistochemistry studies, express MDR1/gp170. These observations strongly suggest that many breast tumors already acquire MDR1/gp170 expression before clinical detection. Among previously untreated breast cancer patients, 20%-55% obtain a worse than PR when they are treated with cytotoxic chemotherapy. Furthermore, among those who respond to first-line chemotherapy, the median duration of response is relatively short (only 5-13 months) (*2*).

These observations are consistent with the likely acquired expression of low levels of MDR1/gp170 and/or other multiple drug resistance mechanisms early in the biology of breast cancer progression. This acquisition may not require the selective pressure imposed by chemotherapy. It also is not clear whether the expression detected after chemotherapy represents either an induced expression or a selection that increases the proportion of MDR1/gp170-expressing tumor subpopulations.

A few studies (*58,66,106*) have looked for MDR1/gp170 expression in the normal breast. The results of these studies suggest that expression is low, absent, or predominantly stromal. Pavelic et al. (*106*), using four independent antibodies, detected MDR1/gp170 expression in normal ductal epithelia. Greater than 80% of normal breast ductal epithelium stained positively with the MRK-16 anti-gp170 antibody, and staining was confined to the luminal surface (*79*). These observations suggest that some MDR1/gp170-positive breast tumors may arise in those previously untreated patients with tumors of intraductal origin.

Selection against MDR1/gp170-related drugs in vitro frequently induces MDR1/gp170 expression (*10*). Furthermore, the meta-analysis results demonstrate that MDR1/gp170 expression is twice as likely to be detected in tumors from treated versus untreated patients. This increased incidence of detectable MDR1/gp170 expression may be a direct consequence of the cytotoxic drug therapy.

Association of MDR1/gp170 Expression With a Worse Than PR

Because of its ability to function as a drug efflux pump, gp170 confers multidrug resistance in vitro. Thus, it would be predicted that the detection of its expression in breast cancer would be associated with clinical resistance. In our analysis, patients with detectable levels of MDR1/gp170 were three times more likely to exhibit a less than partial clinical response to cytotoxic chemotherapy. Since the incidence of MDR1/gp170 expression increases with treatment, and the response rate to second-line chemotherapy is worse than that to first-line treatment, we would expect MDR1/gp170 expression measured after treatment to show a stronger association with a poor clinical response. The post-treatment analysis for all drugs indicated that MDR1/gp170 expression in treated tumors was associated with a fourfold increase in risk for a patient having a worse than PR. These data infer that the expression of MDR1/gp170 may already have been present in the tumors prior to and/or during chemotherapy. Furthermore, they demonstrate a strong association between detectable MDR1/gp170 expression and a poor clinical response to cytotoxic chemotherapy.

There were only limited data concerning the predictive utility of MDR1/gp170 expression measured before treatment. However, in the five evaluated studies (*47,57,61,64,74*), there is a marginally significant 50% increase in the probability of an associated worse than partial clinical response. While this finding suggests that de novo gp170 expression also may play a role in drug resistance, it is inconclusive. The relatively small number of patients and the differences in immunohistochemistry-based assay techniques across studies may contribute to the inability of these studies to demonstrate clearly, either way, the role of gp170 expression in previously untreated breast cancer patients. Additional prospective, randomized, clinical trials are clearly needed to resolve this issue.

Expression of MDR1/gp170 and Overall Survival

The data relating MDR1/gp170 expression to recurrence-free and overall survival could not be analyzed because the required data were not available in most published reports. The individual studies were essentially equally grouped in favor of and against an association between expression and survival. This is not entirely unexpected, since response to therapy generally is not associated with survival (*107,108*). Until sufficient studies are completed and the data are reported in a way that can be readily accessed, any association of MDR1/gp170 expression with survival, whether positive or negative, will remain unknown.

Expression of MDR1/gp170 and Other Prognostic Attributes

Expression of MDR1/gp170, rather than being associated with treatment, may merely reflect a more aggressive phenotype (*89*). Individual studies have found an association between the detectable expression of MDR1/gp170 in breast tumors and a poor prognosis (*61*), poor survival (*61,69*), lymph node metastasis (*109*), and low progesterone receptor expression (*54*). Among all the studies that have examined associations with other prognostic attributes, however, there is little evidence to support the contention that MDR1/gp170 expression is merely a surrogate for other indicators of an aggressive phenotype. We found no association of MDR1/gp170 with either positive lymph nodes, tumor size, grade, histology, or estrogen receptor expression. However, relatively few studies examined such associations. The most commonly evaluated markers, lymph node status and tumor size, were examined in nine studies (*54,57,60,61,63,69,70,74,75*) and seven studies (*54,60,61, 63,70,74,75*), respectively.

Establishing the Functional Relevance of MDR1/gp170 Expression—Considerations and Future Directions

The meta-analysis data clearly indicate that MDR1/gp170 is expressed in a significant proportion of breast tumors. A direct examination of the clinical relevance of MDR1/gp170 expression is clearly the next most important step. The precise delineation of this role is likely to be difficult and complex. Clinical studies have almost exclusively assessed MDR1/gp170 expression either before or after therapy, but not during treatment. In experimental models, MDR1/gp170 expression is frequently induced by exposure to a substrate (*110*), and expression becomes constitutive only after repeated or prolonged in vitro exposure. Thus, in some tumors, expression may be detectable only during either therapy or the later cycles of therapy. The level of expression before or after treatment may underestimate the level induced during treatment and may produce a false-negative impression with regard to the role of MDR1/gp170.

Studies that have begun to address gp170 function in patients have used response to a combination chemotherapy regimen as their end point. This may be a suboptimal approach. Determining the individual contribution of each drug to the response in a specific tumor is currently impossible. For example, when a regimen is used in which each drug is effective as a single agent,

e.g., CAF, what proportion of the response, or lack thereof, will be due to the gp170 substrate? Furthermore, doxorubicin is the major or sole gp170 substrate in chemotherapy regimens used in most studies. However, doxorubicin is subject to several drug resistance mechanisms unrelated to gp170, including an altered expression of manganese superoxide dismutase (*7*) and increased activities of glutathione transferase and topoisomerase II (*111,112*). Thus, when using doxorubicin as the gp170 substrate, lack of a clinical response can be attributed only cautiously to gp170. This is also a concern for the present meta-analysis and suggests that patients previously treated with a non-MDR1/gp170-related regimen should be excluded from future studies, since this treatment may induce cross-resistance mechanisms to some MDR1/gp170-related drugs.

Suggestions for Design of Future Clinical Trials

We wish to raise several issues for consideration, since these issues are likely to have contributed to the significant heterogeneity apparent in previously published studies. We hope that these suggestions will provide some degree of consistency within, if not among, future studies. Several groups (*94,95*) have recently attempted to derive specific guidelines for the assessment of MDR1/gp170 positivity. Our analysis of the sources of heterogeneity among published studies supports some suggestions raised in these reports and raises the following additional issues for consideration:

1) In general, well-designed prospective studies with adequate statistical power are preferable to retrospective studies. Thus, MDR1/gp170 expression should be measured before the commencement of cytotoxic chemotherapy. If a neoadjuvant design is adopted, investigators should consider the feasibility of also measuring MDR1/gp170 expression in biopsy specimens obtained during chemotherapy. Any tissue removed, or accessible either after treatment or upon relapse, also should be examined for MDR1/gp170 expression.

2) Where possible, the study population should comprise patients not previously treated with any systemic therapies. These patients may have fewer other endogenous cross-resistance mechanisms present, e.g., altered topoisomerase or glutathione transferase expression, or at least express other mechanisms at relatively low levels.

3) Measuring gp170 expression may be preferable to measuring MDR1 expression, since immunohistochemistry is more sensitive and can discriminate between stromal and tumor cell expression.

4) Where immunohistochemistry is adopted, there should be a clear definition of positivity and negativity of expression compared with well-defined positive and negative controls. Since some anti-gp170 antibodies cross-react with epitopes on proteins other than gp170 (*58,84,87*), more than one antibody with nonoverlapping cross-reactivities should be considered. Samples should be screened and scored in a blinded fashion by more than one pathologist using criteria established before the analysis. Experimental criteria discussed in detail by others (*94-98*) also should be considered.

5) When the data are presented, the methods of accruing patients (samples), characteristics of the study population, and the treatments (exposures) should be described in detail. Data should be presented so that MDR1/gp170 positivity can be described among the following patient subgroups: those whose tumors are assayed before or after treatment, those whose tumors are treated with MDR1/gp170-related or non-MDR1/gp170-related substrates, and those whose tumors are primary or metastatic.

MDR1/gp170 expression might be associated with the induction of other drug resistance mechanisms. It is possible that exposure to cytotoxic drugs induces a cellular stress response that co-induces several stress and detoxification mechanisms. This response could include increased expression of the heat shock proteins (*113*), glutathione transferases (*5*), and superoxide dismutases (*7*). The activity of some DNA repair processes, including the activity of the topoisomerases, also may be altered (*8*). Since each subpopulation of cells within a tumor may express a different pattern of these various resistance mechanisms, future studies should determine whether MDR1/gp170 expression either is a general marker for drug resistance or arises independently of other resistance mechanisms. A recent study (*114*) suggests that combining assessments of MDR1/gp170 with those of MRP provides a more accurate predictor of clinical drug resistance in patients with acute myeloid leukemia.

Studies with agents that can reverse gp170 function provide another approach to determining the functional relevance of expression. However, this approach is likely to be complicated by several additional factors. While there is no clear consensus on how best to detect MDR1/gp170, several studies with reversing agents (*115-118*) have been performed on breast cancer patient populations in whom the MDR1/gp170 status is either unknown or not reported. Antibodies and complementary DNA probes have been available for several years, and such study designs are discouraged.

One "intrapatient" study design has been suggested that might alleviate some of these concerns and is worthy of consideration (*119*). Previously untreated breast cancer patients with comparable disease status could receive a single MDR1/gp170-related substrate regimen until relapse. These patients are then screened for MDR1/gp170 expression in accessible lesions, and patients with MDR1/gp170-positive tumors are assigned to subsequent groups where the same drug is administered with the reversing agent. The pharmacokinetics of the cytotoxic agent would be established in each patient, both when the drug is administered alone and subsequently when the drug is given with the reversing agent. This will enable each patient essentially to act as his or her own control and reduce the effects of interpatient variation (*119*). To control for unrelated effects on clinical response and pharmacokinetics, selecting patients with broadly comparable performance status may be necessary. Patients should be evaluated for MDR1/gp170 expression before any systemic therapy is initiated.

Implications of This Analysis for Future Translational Research Studies

The studies reviewed in this analysis varied substantially with respect to the level of information presented in the published reports. Because of the nature of translational research, it is vital that researchers provide the information necessary to readily understand the clinical implications of a particular study. Results should be presented in a way that allows differences in the critical subgroups to be discerned, particularly when the activity of the molecule is thought to vary with clinical attributes, e.g.,

expression levels in patients before and after treatment. A clear description of the patient population and method of accrual also is important. Tumor samples assayed in a particular study will rarely come from randomized clinical trials, so it is important to consider the selection factors, implicit or explicit, that influence the study sample's composition. Tumor specimens accrued to tumor banks or to individual studies generally represent a number of clinical and institutional factors, including tumor size, referral patterns, tissue-handling procedures, procurement policies, consent procedures, other protocols competing for tissue, and recruitment into clinical trials. All these factors can influence the biologic characteristics of patients or specimens comprising a particular study.

Conclusions

The data from our meta-analysis indicate that many breast tumors express detectable levels of MDR1/gp170. There is considerable heterogeneity across studies of MDR1/gp170 expression, resulting partly from the different techniques and end points utilized. The incidence of expression is higher in patients who have received cytotoxic chemotherapy and in those who either will have or have had a worse than partial clinical response to chemotherapy. While the functional relevance of this expression remains to be established, these data are strongly supportive of a likely role for MDR1/gp170 in conferring clinical resistance to gp170 substrates in a significant proportion of breast tumors. We found no evidence to support the assumption that MDR1/gp170 expression has no role in breast cancer.

While the precise role of MDR1/gp170 in breast cancer remains to be established definitively, it seems likely that, in tumors where expression is detectable, this expression contributes to the multidrug-resistant phenotype. However, it seems equally likely that multidrug resistance in breast cancer is a multifactorial phenomenon and may include MRP and the altered expression of superoxide dismutases, glutathione transferases, heat shock proteins, and other resistance genes. The precise contribution of each potential multidrug resistance mechanism is unclear, and it is likely that more than one mechanism can operate within the same tumor cell subpopulation and/or within different subpopulations of the same tumor. If correct, then the establishment of a role for any of these other resistance mechanisms will become as complex and controversial as is the study of MDR1/gp170.

The potential complexity that applies to the study of drug resistance in breast cancer likely also applies to other solid tumors. Clearly, the design of future clinical trials to establish the functional relevance of drug resistance mechanisms will require careful and detailed consideration of the patient population, the drugs used to induce response, and the potential contribution of other drug resistance mechanisms.

One purpose of meta-analyses is to identify potential directions for future studies and, if possible, to provide suggestions for incorporation into improved study designs. In this respect, the issues we have raised regarding study design are provided in the hope that they will both raise awareness and generate some discussion of the complexities and difficulties associated with establishing the role of MDR1/gp170 and other drug resistance mechanisms.

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Notes

Supported in part by Public Health Service grants P30CA51008 and P50CA58185 (R. Clarke and B. J. Trock) from the National Cancer Institute, National Institutes of Health, Department of Health and Human Services; and in part by grant RP950649 (R. Clarke) from the Department of the Army, U.S. Army Medical Research and Materiel Command.

Manuscript received November 7, 1996; revised March 25, 1997; accepted April 18, 1997.

Diseases of the Breast, 2nd ed.,
edited by Jay R. Harris.
Lippincott Williams & Wilkins, Philadelphia © 2000.

CHAPTER 22

Animal Models

Robert Clarke and Michael D. Johnson

GENERAL PRINCIPLES

Animal models of breast cancer have been widely used for many years and have contributed significantly to our understanding of breast cancer biology and to the development of several new therapeutic strategies. Because the number of species that develop spontaneous breast tumors is limited, there are few good animal models of spontaneous breast cancer. For example, in addition to rats and mice, mammary tumors also arise spontaneously in dogs,[1,2] but the cost of these models is generally prohibitive. The majority of experimental animal models of breast cancer are limited to the rodent species. However, several different groups of rodent models are available for experimental breast cancer research. These include chemically induced rat mammary carcinomas, virally induced mammary tumors, human tumor xenografts, and transgenic mouse models.

Many aspects of experimental breast cancer research require the use of an appropriate animal model. For example, reproducing the complexity of the endocrinologic environment of the pituitary-adrenal-ovarian axis is beyond the scope of current *in vitro* technologies. Tumor-host interactions, including immunologic, vascular, and stromal effects, and host-related pharmacologic-pharmacokinetic effects, also are relatively poorly modeled *in vitro*. However, even a well-justified requirement for the use of living animals imposes several ethical and scientific considerations. Investigators must give appropriate consideration to the health and welfare of experimental animals (e.g., by providing adequate diet, space, health monitoring, and hygiene).[3] Many of these concerns are of more importance than is often realized. For example, almost all mammary animal tumor models are sensitive to (i.e., inhibited by) caloric restriction.[4–6] Sufficient numbers of animals must be used to provide adequate statistical power and to ensure the validity of the study[7,8] but not such that there is unnecessary animal use.

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Each of the rodent models has its own advantages and disadvantages, and a clear understanding of the limitations and use of each model is critical for its appropriate application. In general, the major models for spontaneous breast cancer are the mouse strains that are susceptible to mouse mammary tumor virus (MMTV)–induced mammary neoplasia and some transgenic mouse models. In these models, the mammary glands potentially express the transforming genes from early life onward (MMTV/neonatal; transgenics/fetal). For the chemically induced tumors, initiation events are induced by the carcinogen. The spontaneous and chemically induced models are particularly useful for chemoprevention studies, because full transformation of the gland has either not occurred (young transgenic and MMTV-infected mice) or occurs within a reproducible time after carcinogenic insult (chemically induced tumors). In the human tumor xenografts, the malignant tissue is directly inoculated into host tissues. Thus, effects on early events (i.e., initiation) are not amenable to study. However, these xenografts provide a good model for the study of malignant progression in the human disease and the screening of drugs and therapies against established human tumors. A major advantage of the xenografts is their human breast cancer origin, whereas a disadvantage of the rodent mammary models is their nonhuman origin. Choice of the appropriate model and a realistic assessment of its limitations are critical for adequate and appropriate experimental design. Siemann[9] made a simple but important observation when he stated that a critical consideration is to ". . . choose the model to address the question rather than force the question on the tumor model."

CHEMICALLY INDUCED RODENT MAMMARY TUMORS

The mammary glands of several rat strains are susceptible to transformation by chemical carcinogens, most notably Sprague-Dawley,[10] Buf/N,[11] Fischer 344,[12] Lewis,[13] and, to a lesser extent, Wistar-Furth.[12] Other strains are relatively resistant (e.g., the Copenhagen rat[14]). The genetic reasons for this resistance are unknown, but resistant strains appear to inherit a dominant autosomal allele on rat chromosome 2[15] that specifically inhibits the progression, but not initiation, of mammary

cells.[16] The primary end points measured with these models are changes in tumor latency, incidence, and multiplicity.[17]

These models appear to closely mimic several key components of the human disease. Rats that have completed a full-term pregnancy and lactation, or have been treated with estrogen and progesterone before carcinogen administration, exhibit a reduced incidence of mammary tumors.[10] Pregnancy at a young age also is known to reduce lifetime breast cancer risk in humans.[18] This may reflect an endocrine-induced differentiation that reduces the number of target undifferentiated stem cells.[19] For chemical carcinogens, the dose of carcinogen and age of the rats also are critical. The rats must be virgin, with the optimal age being 40 to 46 days. Chemically induced tumors also are initially estrogen responsive.[20] Progression to a hormone-unresponsive phenotype can occur rapidly in a significant proportion of tumors. Loss of hormone responsiveness also can occur in human breast tumors. Many of the mammary epithelial tumors that arise are well-differentiated adenocarcinomas. These are histologically similar to a significant proportion of the lesions that arise in the human breast. However, other tumors also can arise with 7,12-dimethylbenz[*a*]anthracene (DMBA) treatment, and it is important for investigators to confirm the origin of any tumors that arise in the mammary fat pad areas. Several excellent reviews are available that describe the comparative biology of these models.[10,21,22]

These mammary tumor models have been in constant use since their description by Huggins and associates in 1961,[23] and their use has provided critical insights into several aspects of breast cancer biology. For example, chemically induced tumors have been used to demonstrate the antitumor and chemopreventive effects of endocrine agents[24–26] and vitamins.[27] Perhaps the most notable example of the use of chemically induced rodent models is their role in the preclinical development of the antiestrogen tamoxifen.

Chemically induced mammary tumors exhibit a low metastatic potential.[28] Although some local invasion is apparent and occasional metastases have been reported, these are rarely sufficiently reproducible to provide a useful model of metastasis. The majority of tumors are initially prolactin dependent,[20] but a similar central role for prolactin in human breast cancer is not currently evident.[29] These characteristics and the high level of *ras* activation [e.g., *N*-nitrosomethylurea (NMU)-induced tumors] limit their applicability for some studies. In general, investigators should be cautious in designing experiments in which agents are coadministered with a chemical carcinogen, because effects on the carcinogen's pharmacokinetics can produce potentially artifactual observations that are specific for the carcinogen used.

The choice of carcinogen may depend to some degree on the study design. When coadministration of the test treatment or manipulation is required, NMU is likely to be a better choice, because it eliminates potentially confounding effects on metabolic activation. When cellular signaling is under investigation, DMBA may prove more useful, because the incidence of activate *ras* mutations is much lower. Interestingly, transformation of HBL100 cells (normal human breast epithelial-derived cells) by DMBA, but not NMU, is associated with an increased expression of basic fibroblast growth factor (FGF).[30] This suggests that cellular signaling is different in DMBA- than in NMU-induced mammary tumors. FGFs have been implicated in breast carcinogenesis.[31] For many studies, the choice of carcinogen is unlikely to significantly affect the outcome or interpretation of the studies.[32]

7,12-Dimethylbenz[*a*]anthracene

DMBA is a potent inducer of mammary carcinomas. It is generally administered by oral gavage, frequently as a solution in peanut oil. Generally within 10 to 15 weeks, 20 mg per animal produces a final incidence of 100% adenocarcinomas. The mammary tumors arise in the epithelium of the terminal end buds, which are comparable structures to the terminal ductal lobular unit in the human breast.[10] The tumors are generally ductal carcinomas, papillary carcinomas, and intraductal papillomas.[10] The comparative biology of DMBA-induced mammary carcinomas has been extensively reviewed by Russo and associates.[10,21]

DMBA is highly lipophilic and requires metabolic activation for its carcinogenicity.[32] Several tissues are capable of activating DMBA, and these include the mammary gland. However, there also is extensive hepatic activation, and some of the ultimate carcinogens may be systemically active. Both liver and mammary activation may be important in these models. The potential importance of hepatic metabolism is evident from the observation of reduced mammary tumorigenesis in animals in which glucuronidation is blocked.[33] Hepatic but not mammary DMBA activation is inhibited by dietary butylated hydroxytoluene, and this is sufficient to reduce DMBA binding to mammary gland DNA.[34] The ability of direct administration of DMBA to the mammary gland to induce mammary tumors provides clear evidence of the likely importance of its activation within the gland.[21,35] Primary cultures of rat mammary epithelial cells also are able to metabolize DMBA.[36]

Coadministration of agents that alter either its lipid biodisposition or its hepatic/mammary activation can influence subsequent tumor incidence. However, these apparent effects on tumorigenicity can be considered artifactual, because they are pharmacologic effects specific to the carcinogen. The potential for such artifacts requires careful experimental study design when DMBA is used, for example, in studies with agents that could alter hepatic function or in dietary studies that use high fat.[37] The metabolism of DMBA was reviewed in 1997.[32]

N-Nitrosomethylurea

The ability of NMU to produce mammary tumors was reported by Gullino and associates[38] in 1975. NMU induces

mammary carcinomas in rodents when administered subcutaneously or intravenously at 50 mg per kg. Tumor incidence and latency are comparable to that observed with DMBA administration and also exhibit steroid hormone and prolactin responsiveness.[39] Because NMU does not require metabolic activation, there are fewer concerns regarding coadministration artifacts than for DMBA. However, approximately 75% of rodent mammary tumors induced by NMU exhibit altered *ras* expression/activation[11] that occurs during initiation.[40] The incidence of altered *ras* expression in human breast cancer is approximately 20% and represents rare alleles or slight overexpression, or both.[41] Furthermore, its role in human breast cancer initiation, promotion, progression, or a combination of the three remains unclear.[41,42] This contrasts with the high frequency of *ras* mutations observed in the NMU-induced rodent tumors.[11,40]

The high incidence of *ras* activation potentially reduces the use of NMU-induced tumors for signal transduction-mechanistic studies, because there is a high probability that *ras*/G protein–mediated pathways will predominate. The high incidence of activated *ras* increases the likelihood that data from such mechanistic studies could be heavily skewed. Although this also limits studies of the ability of agents (e.g., tumor promoters) to further increase the incidence of *ras* expression, it may prove to be a good model for studying treatments that could either reduce *ras* expression or use *ras*-mediated signal transduction pathways.

Activation of *ras* may not be the primary effect of NMU. It has been suggested that NMU-induced tumors arise in cells that already possess the activated *ras*.[43,44] One possible interpretation is that NMU either promotes the proliferation of these cells or it induces additional mutations that generate the fully transformed genotype, or both.

VIRALLY INDUCED RODENT MAMMARY TUMORS

Mouse Mammary Tumor Virus–Induced Tumors

Several mouse strains are susceptible to infections that subsequently produce mammary tumors (e.g., C3H, CD1, RIII, GR, SHN, BR6). Neonatal female mice are infected with MMTV through their mother's milk. Infected female mice of susceptible strains develop preneoplastic hyperplastic alveolar nodules that are generally apparent from 4 weeks of age. In C3H/OuJ[6] and C3H/HeJ mice,[45] mammary tumors begin to appear at 24 to 28 weeks of age. An approximately 50% incidence in mammary tumors is achieved in virgin mice by approximately 35 weeks of age.[6] Many of the MMTV models exhibit a strong pregnancy- or progesterone-dependent increase in incidence.[46] In common with the chemically induced rat mammary tumor models described previously, these models also exhibit a strong prolactin dependence[45] and are responsive to retinoids.[46] Both oophorectomy and treatment with tamoxifen also induce regression in these spontaneous mammary tumors.[46] However, the histology of many of these mammary tumors is not clearly comparable to that of the human disease. Atypical lobular type A lesions appear similar to the hypoplastic alveolar nodules that arise in susceptible, MMTV-infected mouse strains.[47] Metastasis occurs in many infected animals, with the lungs being a major metastatic site.[48]

The transforming potential of MMTV is almost certainly the result of virally induced mutational insertion.[49] MMTV proviral insertion can alter the expression of several genes. These include *int-1/Wnt-1* (mouse chromosome 15), *int-2*/FGF-3 (mouse chromosome 7), *int-3* (mouse chromosome 17; human 12q13), *HST/FGF4* (mouse chromosome; human 11q13), *Wnt-3* (mouse chromosome 11; human 17q21-22), *int-6* (mouse chromosome 15; human 8q22), *Wnt-10b* (mouse chromosome 15; human 12q13), and *FGF8* (mouse chromosome 19; human 10q).[48] The most common insertions are observed at the *int-1* and *int-2*/FGF-3 loci.[49] Different transcriptional/translational start sites and polyadenylation sites in different tissues can produce expression of various *int-2*/FGF-3 messenger RNA (mRNA) species. However, each of these mRNAs can produce the same protein (reviewed in reference 49). Although amplification of *int-2*/FGF-3 and *hst*/FGF-4 is observed in both human and mouse mammary tumors (e.g., approximately 30% of human breast tumors), their respective mRNAs and proteins are rarely expressed in the human disease.[50–52] MMTV-induced oncogene activation has been reviewed in detail.[48,49]

Polyoma-Induced Tumors

Mouse polyoma virus can induce mammary tumors when infection occurs neonatally and when infection is present in immunodeficient hosts. Mammary hyperplasia, dysplasia, and mammary tumors are observed in female mice infected with the polyoma WTA2 virus at 6 weeks of age.[53] Infected mammary glands exhibit an initial epithelial hyperplasia, followed by dysplasia 6 weeks *post inoculum*. Glands ultimately develop mammary adenocarcinomas of ductal origin (100% incidence) by 6 to 9 weeks *post inoculum*. The middle T antigen of the virus also has been successfully used to generate a transgenic mouse mammary tumor model. All of the polyoma virus–associated models produce tumors that are histologically comparable to mammary ductal adenocarcinomas in humans. Unlike the MMTV and chemically induced rodent models, the polyoma-induced tumors are ovarian independent.[53] Interested readers can find an excellent review of this model by Fluck and Haslam.[54]

Adenovirus-Induced Tumors

One-day-old Wistar-Furth rats (<24 hours of age) inoculated subcutaneously with human adenovirus type 9 develop benign mammary fibroadenomas, phyllodes-like tumors, and solid sarcomas.[55,56] Palpable mammary lesions develop by 3 to

5 months of age, with the benign lesions apparently of primarily mammary fibroblastic origin, as determined by expression of type IV collagen and vimentin. Unlike the other rodent mammary tumor models, the areas of neoplasia are of myoepithelial and not epithelial origin, as indicated by their continued expression of type IV collagen, vimentin, and muscle-specific actin. The tumors are estrogen responsive, as indicated by an oophorectomy-induced inhibition of tumor development, induction by diethylstilbestrol, and the presence of estrogen receptor (ER) mRNA.[55] The adenovirus tumors provide a novel and useful model of mammary fibroadenomas, which are relatively common benign human breast lesions. This model has been reviewed in detail by Javier and Shenk.[56]

HUMAN TUMOR XENOGRAFTS

The xenografting of human tumors into athymic nude mice has become almost routine. However, the nude mouse is not the only immune-compromised rodent available. Mutations at approximately 30 loci have been shown to produce reduced immune function in mice.[57] The major mutations used to generate hosts for xenografts are the nude (*nu*), beige (*bg*), severe combined immunodeficiency (*scid*), and X-linked immunodeficiency (*xid*). Of these, the *scid* mouse is generally considered to exhibit the greatest degree of immunosuppression. The combined *bg/nu/xid* mutation strain (e.g., NIH III) also produces severely immunocompromised animals but has received less attention, perhaps as a result of a clotting disorder that reduces their use in studies that require survival surgery.

Most investigators report a relatively low take rate when human breast tumor biopsies are xenografted directly into immune-compromised rodents. This low rate generally reflects the frequency with which cell lines or continuous xenografts can be established. For many purposes, slow-growing tumors are of limited value, and primary tumors with long doubling times have often been discarded. Sakakibara and associates[58] report that approximately 25% of primary breast tumors, when xenografted into *scid* mice, exhibited a sufficient growth rate to allow for repeated passage (i.e., tumors reached a 2- to 3-cm diameter within 6 months). Metastasis to lung or other sites, or both, was observed in 8 of 12 tumors. The generation of reproducibly metastatic models is an important goal, because relatively few well-characterized metastatic models are available. However, new models are occasionally reported. Mehta and colleagues[59] recently described an endocrine-responsive metastatic xenograft (UISO-BCA-NMT-18). It is hoped that the new metastasis models will be fully characterized and sufficiently distributed so that their uses will rapidly become apparent.

Human breast cancer cell lines inoculated into nude mice represent the majority of human breast tumor xenograft models. However, relatively few xenografts have been in regular and widespread use other than MCF-7 (endocrine-responsive) and MDA-MB-231 (endocrine-unresponsive) cells. In part, this reflects the low success rate for establishing human breast tumors either directly as xenografts or as stable established cell lines *in vitro*.

Despite the ability to apply selective pressures that result in variants with altered endocrine responsiveness,[60–62] the majority of endocrine-responsive xenografts are phenotypically stable, at least with respect to biologically important characteristics (e.g., tumorigenicity, steroid hormone receptor expression, hormone responsiveness). We have not observed any spontaneous loss of estrogen dependence in MCF-7 cells (estrogen receptor positive, estrogen dependent), gain of estrogen responsiveness in MDA-MB-435 cells (estrogen receptor negative, estrogen unresponsive), or alteration in estrogen responsiveness of MCF7/MIII cells (estrogen receptor positive, estrogen independent, estrogen responsive) maintained routinely in our laboratory in the absence of selective pressures. Indeed, the major phenotypic characteristics of hormone responsiveness, hormone receptor expression, antiestrogen responsiveness, tumorigenicity, and metastatic potential remain remarkably stable in the majority of human breast cancer cell lines. The stability of human tumor xenografts is widely reported (reviewed in reference 63). Some minor phenotypic diversity is observed between laboratories and is not surprising, because some of these cell lines have been in continuous culture for more than 15 years. Nevertheless, these models have the advantages of being human in origin and relatively reproducible with regard to their endocrine responsiveness and metastatic potential. A description of the characteristics of the major xenografts is provided in Table 1.

Many studies of endocrine agents, or of use of endocrine-responsive xenografts, are performed in oophorectomized mice. The levels of circulating estrogens in these animals are very low and approximate the levels found in postmenopausal women.[64–66] Because the major endocrine-responsive human breast cancer cell lines (e.g., MCF-7, ZR-75-1, T47D) were derived from tumors in postmenopausal women,[67] the endocrine environment of the oophorectomized mouse is appropriate. There also is increasing evidence that orthotopic implantation produces tumors with a more biologically relevant phenotype and greater tumor take rate.[68–71] Despite potential differences between the rodent mammary fat pad environment and the human breast,[10] the mammary fat pad provides an appropriate orthotopic site that is readily accessible. Although most human breast cancer cell lines grow adequately in almost any subcutaneous site (e.g., the flank is widely used), inoculation into the mammary fat pad is the preferred site. The incidence of metastasis from solid breast tumors is higher when the primary tumor is orthotopic rather than subcutaneous.

Endocrine-Responsive Xenografts

Relatively few human breast cancer xenografts exhibit an endocrine-responsive phenotype, and all are estrogen receptor

TABLE 1. *Characteristics of representative transplantable mammary tumor cells*

Cell line	Origin/derivation	Estrogen responsiveness	Invasive/metastatic	References
MCF-7	Human breast cancer cell line	Dependent	–/–	73,184
ZR-75-1	Human breast cancer cell line	Dependent	–/–	185,186
T47D	Human breast cancer cell line	Dependent	–/–	185,186
MCF7/MIII	MCF-7 variant	Independent/stimulated	+/±	60,73
MCF7/LCC1	MCF-7 variant	Independent/stimulated	+/±	73,79
MCF7/LCC2	MCF-7 variant	Independent/stimulated	ND	61
MCF7/LCC9	MCF-7 variant	Independent/stimulated	ND	187
MCF7/MKS-1	MCF-7 transfected with FGF-4	Independent/inhibited	+/+	183
MLα	MDA-MB-231 transfected with ER	Independent/inhibited	ND	87
T61	Human xenograft	Independent/inhibited	ND	188,189
NCI/ADR-RES (previously MCF-7ADR)	Selected for doxorubicin resistance. It is no longer clear that these cells are of MCF-7 origin	Independent/unresponsive	–/–	190,191
MDA-MB-435	Human breast cancer cell line	Independent/unresponsive	+/+	86
MDA-MB-231	Human breast cancer cell line	Independent/unresponsive	+/+	86
Hs578T	Human breast cancer cell line	Independent/unresponsive	+/±	185

ND, no data available; +, phenotype observed reproducibly; –, phenotype rare; ±, phenotype observed occasionally.

positive. There are two categories of endocrine-responsive cells, (a) estrogen dependent and (b) estrogen independent and estrogen responsive.[72] The estrogen-dependent xenografts do not form proliferating tumors in the mammary fat pads of oophorectomized immunodeficient mice without estrogen supplementation, generally in the form of a 60-day release, 0.72-mg 17β-estradiol pellet placed subcutaneously in the interclavicular region. Examples of estrogen-dependent xenografts include the MCF-7, ZR-75-1, and T47D cell lines. Most of the estrogen-responsive xenografts produce relatively well-differentiated adenocarcinomas,[73] are inhibited by tamoxifen,[74–78] and are poorly invasive and nonmetastatic.[73]

Several estrogen-independent and estrogen-responsive variants have been derived from estrogen-dependent cells in the authors'[60,61,79] and other laboratories.[62] These variants form proliferating tumors in oophorectomized immunodeficient mice without estrogen supplementation. However, they grow more rapidly in the presence of an estrogen pellet.[60,79] Examples of estrogen-independent and estrogen-responsive xenografts derived from MCF-7 cells include MCF7/MIII,[60] BSK-3,[60,62] MCF7/LCC1,[79] and MCF7/LCC2 (tamoxifen resistant).[61]

Analysis of the growth and endocrine responsiveness of the various endocrine-responsive xenografts has provided useful information on the biology of malignant progression[80,81] and cross-resistance among antiestrogen therapies.[61,72] For example, the ability to isolate estrogen-independent cells from estrogen-dependent cells indicates a possible progression pathway to acquired estrogen independence in breast tumors that arise in postmenopausal women.[72,80,82]

MCF7/LCC2 cells are resistant to the inhibitory effects of 4-hydroxytamoxifen when growing both *in vitro* and *in vivo*.[61] However, MCF7/LCC2 cells are not cross-resistant to the steroidal antiestrogens ICI 182,780[61] and ICI 164,384.[83] These data would predict that patients in whom tamoxifen has induced a response but subsequently failed would respond to a steroidal antiestrogen. Preliminary data from a phase I trial of ICI 182,780 now demonstrate responses in patients in whom tamoxifen has failed.[84] Thus, the pattern of antiestrogen responsiveness exhibited by the MCF7/LCC2 cells accurately predicted for a previously unknown pattern of clinical response.

MCF-7 human breast cancer cells transfected with an expression vector directing a high constitutive expression of FGF-4/kFGF produce highly vascular tumors that are inhibited by physiologic doses of estrogen and stimulated by pharmacologic doses of tamoxifen.[31,85] The inverse response is exhibited by the parental MCF-7 cells.[77,78] However, these tumors produce a high incidence of lung and lymphatic metastases. Estrogen receptor–negative (estrogen unresponsive) and metastatic MDA-MB-231 cells[86] transfected with the estrogen-receptor gene also exhibit an estrogen-inhibited phenotype.[87] These cells may provide potentially novel breast cancer metastasis models. MCF-7 tumors selected *in vivo* for resistance to tamoxifen also exhibit a tamoxifen-stimulated/estrogen-inhibited response pattern.[74,78]

The relevance of the inverted endocrine responsiveness of these models to the human disease is not immediately apparent. Tumors inhibited by physiologic estrogen concentrations may not arise frequently, because most breast tumors appear to contain physiologic concentrations of estrogens, irrespective of menopausal status.[88] Pharmacologic doses of estrogens produce remissions in hormone-responsive breast tumors,[89,90] and there is no clear evidence that either physiologic estrogen doses or hormone replacement therapy pro-

duces remissions in postmenopausal patients with breast cancer. Indeed, hormone replacement therapy is associated with a modest increase in the risk of breast cancer.[18] However, these models do suggest that withdrawal responses to tamoxifen may occur more frequently than has been previously reported. Despite the estimation that the total exposure to tamoxifen between 1971 and 1988 was more than 1.5 million patient-years,[91] and is now in excess of 8 million patient-years, reports of tamoxifen withdrawal responses are relatively rare[89,92,93] and often represent individual case histories.[94–96] This issue will remain controversial until sufficient clinical trials are conducted to specifically address the actual frequency of withdrawal responses to tamoxifen.

Endocrine-Unresponsive Xenografts

The majority of human breast tumor xenografts are estrogen receptor negative and therefore are estrogen unresponsive. These xenografts also tend to be more locally aggressive and exhibit a significantly increased metastatic potential. Two estrogen-unresponsive xenograft models (MDA-MB-231 and MDA-MB-435) are capable of producing distant metastases in an apparently reproducible manner and with a sufficient incidence to be of use in the study of spontaneous metastasis.[86] The MDA-MB-435 model is sensitive to dietary manipulations.[97,98] We have isolated an ascites variant of these cells (MDA435/LCC6), which is sensitive to a variety of cytotoxic drugs with proven efficacy in the human disease.[99] More recently, we have isolated an estrogen-unresponsive cell line (LCC15-MB) from a bone metastasis.[100,101] Most endocrine-unresponsive xenografts produce poorly differentiated tumors. The MDA-MB-231, MDA-MB-435, MDA435/LCC6, and LCC15-MB cell lines can produce metastases in immunodeficient mice.

Immunodeficient Mouse Models

We have previously reviewed the immunodeficiencies of the most widely used xenograft hosts.[102] These are discussed only briefly here. Mice that are homozygous for the nude (*nu*) mutation are athymic[103] and exhibit a defect in thymic-dependent B-cell maturation but possess apparently normal virgin B cells.[104] Primary responses to T-dependent antigens are low[105] and may be reversed by reconstitution of the mice with T cells.[106–108] Serum immunoglobulin M (IgM) levels are similar to those of their heterozygote littermates.[109] However, there is a significant decrease in the number of cells making IgG and IgA.[107] Despite their ability to maintain human tumor xenografts, nude mice retain considerable immunity. For example, they possess substantially *greater* numbers of natural killer (NK) cells than comparable heterozygotes, and this may contribute to the relatively low incidence of metastases from human tumor xenografts.[110,111] Nude mice possess a relatively normal primary response to T cell–independent antigens,[109] and their splenocytes generate lymphocyte-activated killer (LAK) cells at levels similar to those observed in normal mice.[112] The levels of macrophages are equivalent in *nu/nu* and *nu/+* mice and frequently exhibit tumoricidal properties.[113]

The *scid* mutation is one of the few single mutations that produce viable severely immunodeficient mice. It causes a deficiency in the rearrangement of genes coding for antigen-specific receptors on B and T cells.[114] Pre-B and B cells are absent, and the remaining T cells are nonfunctional. However, myeloid lineage cells appear normal.[115,116] Homozygotes generally have no detectable levels of IgGs 2a, 2b, and 3a[57] and IgA.[117] Some individual animals produce detectable levels of two or more IgG isotypes or IgM, or both.[117] NK cells, macrophages,[118] and LAK activity are comparable to those of normal mice.[112]

Several immunodeficiency mutations have been combined to produce severely immunodeficient animals. The most widely used to date are mice that bear the combined *bg/nu/xid* mutations (e.g., NIH III).[119] The *bg* mutation produces a significant block in NK function.[120,121] This is in marked contrast to the increased NK activity observed in mice that are homozygous for the *nu* mutation.[110,111] The *bg* mutation also produces functional defects in T cells, macrophages, and granulocytes.[57] The *xid* mutation in males (*xid*/Y) and homozygous females produces mice that cannot generate a humoral response to thymus-independent type-II antigens.[57,122] In the *bg/nu/xid* combination, the *nu* mutation produces mice that are deficient in mature T cells; the *xid* mutation produces a deficiency in mature B cells[123,124]; and the *bg* mutation reduces the elevated NK activity conferred by the *nu* mutation.[120,121] However, some NK activity remains detectable.[78,119] The *bg/nu/xid* strain is deficient in B-cell and T-cell activities and cannot produce detectable LAK activity.[119] Mice that are homozygous for *bg* also exhibit a clotting deficiency due to a platelet disorder. This latter characteristic can be problematic in the *bg/nu/xid* mice if the experimental design requires survival surgery (e.g., oophorectomy).

Endocrine Effects on Immune Function

Endocrine treatments are known to modulate immune function both in immunodeficient mice and in humans. Estrogen receptors have been demonstrated on peripheral blood mononuclear cells, splenic cells, thymic cells,[125] and $CD8^+$ T cells.[126,127] Estrogens can alter B-cell function and increase IgM secretion.[128] Inhibition of T-suppressor function and T-helper maturation also have been reported.[129,130] Physiologic concentrations of estrogen stimulate pokeweed mitogen–induced Ig synthesis of B lymphocytes.[129,131] Pharmacologic administration of the progestin medroxyprogesterone acetate results in a reduced $T4^+/T8^+$ ratio, perhaps as a result of its glucocorticoid activity.[132] The progestagen lynestrenol has been reported to stimulate active T rosetting and phagocytosis by monocytes.[133]

The number of macrophages does not appear to be significantly modulated by E2 in nude mice,[134] but there are several reports of altered NK cell activity. Pharmacologic but not physiologic concentrations of E2 inhibit NK activity in athymic nude mice.[134–137] The effect of pharmacologic concentrations of E2 is biphasic; a stimulation of NK activity occurs within the first 30 days, with inhibition being observed thereafter.[135] The delay in detecting an E2-induced suppression of NK activity has been widely reported.[134–137] E2-induced effects on NK activity are unlikely to be responsible for E2's effects on breast tumor cell growth *in vivo*. The majority of breast tumor xenografts produce readily palpable tumors within 10 to 14 days, during which time the already high NK activity in nude mice is further elevated.[135] Furthermore, the concentrations of estrogens reported to suppress NK activity appear to exceed the physiologic concentrations used to stimulate MCF-7 hormone-dependent breast tumor growth in nude mice.[60,64,138–140]

Tamoxifen stimulates NK activity both *in vitro*[141] and *in vivo*.[78] Other antiestrogens can increase pokeweed mitogen–induced Ig synthesis of B lymphocytes.[142] The aromatase inhibitor aminoglutethimide reduces serum estrogens and increases NK activity in patients with breast cancer.[143] Clearly, there are significant interactions between endocrine agents and effectors of cell-mediated immunity. The isolation of breast cancer cell variants with differing responsiveness to both hormonal manipulations and immune response effectors will greatly improve our ability to determine the nature of these interactions and how they can be manipulated to therapeutic advantage. In 1998, we observed that the tamoxifen resistance of the MCF7/LCC2 cell line can be affected *in vivo* by the administration of blocking transforming growth factor (TGF)-β antibodies, which appears to reflect up-regulated secretion of TGF-β sufficient to block NK cell lysis.[144] This suggests that the endocrinologic effects of some agents may have important immunologic consequences for some breast cancers.

The ability of endocrine agents to perturb several effectors of cell-mediated immunity requires careful consideration for study design. For example, the ultimate reduction in NK activity by estrogens (at 30 or more days) depends on the dose of estrogen used and the length of the analysis. Long-term *in vivo* experiments of both more than 6 weeks' duration and using pharmacologic doses of estrogens could be influenced by perturbations in immunologic function. Although there is little direct evidence to the contrary, the relatively low estrogen levels (~300 pg/mL estradiol)[145] released from the widely used 60-day release, 0.72-mg estrogen pellets (Innovative Research of America, Toledo, OH) are probably insufficient to suppress NK activity to a level at which tumorigenicity data would be markedly influenced.

Use of Animal Models in Drug Screening

A major use of the human xenograft models described previously is for the identification of new drugs or evaluation of new drug combinations, or both. In general, relatively few breast cancer models have the characteristics most widely sought, such as rapid and reproducible tumor doubling time. Nonetheless, several models are included in the current National Cancer Institute *in vivo* screen and appear useful in this context. MCF-7 xenografts are often relatively slow growing (compared with the P388 or L1210 mouse models) and generally do not produce lethality within an acceptable time frame. These are among the most widely used xenografts and are particularly helpful in assessing antiestrogenic compounds. Other models in use include the T47D (also endocrine responsive) and the HS578T, BT-549, MDA-MB-231, and MDA-MB-435 models (all endocrine unresponsive).

There are several important considerations in the use of animal models for drug screening, including the choice of xenograft, host, end point, and data analysis. Several of these have recently been described in detail.[7,8] An appropriate experimental design requires some consideration of the nature and quality of the data that are likely to be obtained. One of the more important, but often overlooked, areas is in the choice of numbers of animals per experimental group. Most institutional animal care and use committees now require investigators to provide a realistic estimate of their predicted animal usage before initiation of experimentation. The most effective means to this end is to perform an appropriate statistical power estimation. Group size must be sufficient to enable statistical analysis of the data, and the power estimate should ensure that power is maintained should some animals die from unrelated causes. This issue is discussed in some detail by Hanfelt.[7]

The major end points for assessing drug activity include percentage increased life span and tumor growth delay. Whereas survival studies were a mainstay of anticancer drug evaluation for decades, they are becoming less common, largely due to restrictions in the use of death as an experimental end point. In some cases, it may be possible to substitute morbidity, particularly when the characteristics of the tumor model are well defined and the animals experience reproducible and predictable morbidity before death. This approach can still allow for survival studies while substantially reducing suffering. The most common approach for data analysis is the use of standard survival analyses (e.g., using a Kaplan-Meier approach with the log rank test).

Generally, tumor growth delay studies are much less stressful for the animals but provide essentially comparable estimates of activity to other types of studies. For these studies, tumors are grown to a predetermined size, and animals are randomized into groups so that the mean tumor size is effectively the same in each group. Only proliferating tumors are used, and all selected tumors should have similar tumor doubling times. In principle, growth delay is the difference, among treated and untreated groups, in the times needed for tumors to reach a predetermined size. There are several ways to approach data analysis from such studies. Survival analysis can be used, because these are essentially

time-to-event analyses. Repeated measures of analyses of variance also can be used, particularly if a larger than expected number of treated tumors fail to reach the predetermined size.[7,8] For cytostatic treatments, tumor doubling times can be estimated and compared by analysis of variance or multivariate analysis of variance.[146]

TRANSGENIC AND TARGETED MUTANT MOUSE MODELS

The development of transgenic and targeted mutant mouse technologies has, over the last few years, led to the development of a number of new animal models of breast cancer. The nature of this technology makes these models particularly useful for studying the impact of specific genetic lesions on normal mammary development, carcinogenesis, and tumor progression, and often a single model can be used to study all three processes.

In a typical transgenic model, the mice have been genetically altered so that the expression of a particular gene—c-*myc*, for example—has been altered in some way. This might include increasing the expression of the gene, altering the temporal aspects of its normal regulation, or both. A new strain of transgenic mice is often generated as follows: A DNA expression construct is prepared in which sequences that code for the protein of interest are ligated next to other sequences (the promoter) that will drive the expression of the gene in the manner desired. This DNA construct is then injected into one of the pronuclei of single-cell mouse embryos. The embryos are implanted into the reproductive tract of pseudopregnant female mice that then carry the embryos to term. In a proportion of the resultant pups (approximately 5%), the injected DNA will have become integrated into the genome and be passed to subsequent generations.[147] If the DNA construct has not been rearranged, otherwise damaged during this integration event, or integrated into a part of the genome where expression is suppressed, the promoter drives the expression of the introduced gene. The choice of promoter used to prepare the construct to a large extent determines the level, site, and timing of transgene expression. Various different promoters have been used, and they fall into three basic classes: those that exhibit marked tissue specificity, those that do not, and those in which expression can be regulated by external manipulation.

Although there are transgenic models with mammary phenotypes that have used nontissue-specific promoters,[148] many investigators interested in breast cancer have chosen to use promoters that predominantly direct expression to the mammary tissue. There are a number of such promoters, of which two have proved particularly useful. The first is MMTV, which consists of a portion of the MMTV long terminal repeat (LTR) that directs expression in the adult mouse to the mammary glands, salivary glands, and several other secretory tissues. Studies with MMTV-Cre mice have shown that there can be widespread low-level expression from this promoter, however.[149] The second promoter is the whey acidic protein (WAP) promoter, which directs expression to the mammary gland in a pregnancy- and lactation-dependent manner. High levels of expression are achieved by day 16 of pregnancy, although it can be detected by approximately day 10.[150] Despite this dependence on pregnancy and lactation for maximal promoter activity, in some models there is sufficient expression in virgin animals for a phenotype to develop.[151] Studies with WAP-Cre mice suggest that there is much less low-level expression in nonmammary tissues than is found with MMTV.[149]

Other promoters that have been used include the ovine beta-lactoglobulin promoter, which is expressed predominantly in the mammary gland during lactation, and the rat prostatic steroid-binding protein C3(1) promoter, which directs expression to the prostate and mammary gland.[152] Substantial research is being conducted to develop other mammary-specific promoters, largely with the goal of being able to produce valuable proteins in the milk of farm animals. As these promoters are developed further, they may be useful for making transgenic models of breast cancer.[153] Inducible promoters have also been used in transgenic model systems, specifically a system based around the tetracycline operator protein and a system based on the receptor for the insect hormone ecdysone.[154,155] In both systems, the gene of interest is placed under the control of a promoter that depends for function on the presence of an activator protein (the tetracycline transactivator protein or the ecdysone receptor). In the ecdysone receptor system, expression is then induced by administering a ligand for the receptor. The tetracycline system can be engineered such that administration of tetracycline either induces or represses expression. By placing the gene for the activator protein under the control of a mammary-directed promoter, such as the MMTV promoter, it is possible to obtain inducible expression of the gene of interest in the salivary gland.[156] Although few models thus far have made use of this potentially very useful technology, a study in which the SV40 large T antigen was inducibly expressed in the mammary gland has clearly demonstrated the possibilities of this approach, although expression in the mammary gland has been problematic.[157]

In a targeted mutant mouse model, a specific genetic lesion has been introduced into the genome of the mice. This could include the deletion of a gene (knockout mouse), the introduction of a specific mutation, or the substitution of the mouse gene for the human gene, producing a "humanized" mouse. These models are created by use of pluripotent embryonic stem cell cultures. These cells, when introduced into a mouse embryo, become integrated, resulting in a mosaic animal in which some of the cells are of embryonic stem (ES) cell origin. If the ES cells make up the gametes of the mouse, then any genetic changes within the ES cells are passed on to the next generation. This means that the powerful genetic techniques (e.g., gene deletion, mutation, substitution, or a combination, mediated by homologous recombination) that are only practicable in tissue culture sys-

tems can be applied to animal models. Thus far, the majority of such models have been ones in which a gene had been knocked out, although several humanized models have been created that may prove useful in the study of breast cancer. One problem with the majority of the knockout mice made so far is that the gene is disrupted in all cells from the time of conception. This can lead to problems with embryonic lethality, as is the case with BRCA-1 and -2 knockout mice,[158] or with difficulty in seeing a mammary phenotype, due to the rapid death of the animals caused by another malignancy.[159] The use of the Cre-Lox recombinase system to make genetic alterations *in vivo* that are specific to the mammary gland by expressing Cre using a WAP or beta-lactoglobulin promoter may alleviate both problems in the next few years.[149,160]

Transgenic models have several advantages and disadvantages when compared with other animal models of breast cancer. One of the principal advantages is that these models allow the study of oncogenes and other proteins that are believed to be important in human mammary carcinogenesis. In many transgenic models that use oncogenes relevant to human mammary cancer, the tumors that result are histologically similar to human cancers.[161,162] One feature of some transgenic mammary tumor models can be seen both as a technical advantage and as a potential deficit in terms of relevance to the human disease. In some models, all animals develop tumors with almost exactly the same latency.[163,164] Clearly, this is quite different than the natural history of the disease in humans but can be extremely helpful when these mice are used in cancer prevention studies to assess the impact of some anticancer strategy. Another feature of transgenic systems that has been used very effectively is the ability to cross two transgenic strains, allowing the impact of two defined genetic insults to be examined.[164]

Transgenic models can assist in bridging the gap between *in vivo* and *in vitro* studies, because they can be used as a source of mammary epithelial and mammary cancer cell lines that can then be further studied and indeed reimplanted to form transgenic mammary glands.[165] Transgenic systems can also be used in combination with carcinogens to study the impact of a specific genetic change on the timing and biology of the tumors that are produced. For example, this method has been used to investigate the impact of the presence of mutant p53 on the effects of carcinogen insult.[166]

It has been known for some time that the genetic background of the transgenic strain can have a significant effect on the latency, penetrance, and aggressiveness of the phenotype that a given transgene produces. For example, dramatic differences in the latency of tumor formation are seen when the MMTV-polyoma middle T transgene is bred onto different inbred mouse backgrounds. These systems are being used in attempts to clone modifier genes that may be relevant to the penetrance issues seen in human disease and are simplified by the relative ease of doing genetic studies in mouse models.

Transgenic models of breast cancer are not without their disadvantages. Some of the mammary tissue–specific promoters currently available require parity for expression. However, the endocrine environment of pregnancy and lactation, and the resulting physiologic and morphologic changes that concurrently occur in the mammary gland, can affect phenotype in a manner that does not directly reflect the activity of the expressed gene in nonpregnant or nonlactating mammary tissues. Although expression of some genes can be restricted to the mammary gland, it can be difficult to differentiate among endocrine versus local effects (e.g., autocrine, paracrine) when the transgene is a secreted factor. In many systems, expression of the transgene *in utero* can adversely impact the normal development of the gland. We have previously alluded to the importance of the *in utero* environment in affecting subsequent breast cancer risk. Some transgenes may affect normal gland development, producing a deformed gland from an early age.[167] This is likely to be different than most sporadic human breast cancers, in which oncogene activation presumably occurs as the result of some insult after normal mammary development. The transgenic models may more closely reflect the pattern of familial breast cancer. These gland abnormalities also can present a technical problem, with some female mice being unable to suckle their pups.[152,167] It is likely that the increasing use of regulable transgenic models will circumvent this problem.

An important issue to consider when evaluating a transgenic system is the possibility that the integration of the transgene into the mouse genome may have resulted in some form of insertional mutagenesis. This could be considered analogous to the insertional mutagenesis that is responsible for MMTV-induced neoplasia. This issue is usually addressed by assessing the phenotype of several strains bearing the same transgene in an attempt to ensure that the pathology seen is truly the result of transgene expression.

A further significant difference between most transgenic models and human disease is that the transgene is usually expressed in most or all of the mammary epithelial cells, whereas it is usual to think of the oncogenic event as occurring in one or only a few cells. Tumors can be multifocal, polyclonal, or both. Polyclonality is rare in human breast tumors.[82] However, this also may apply to some of the virus- and chemical-induced models, in which it is likely that many cells are infected or exposed, but not all give rise to tumors.

The concept of these models relies to some extent on the ability of a single gene to produce a fully neoplastic phenotype that would include initiation, promotion, and progression events. It is not clear that cancer is a single-gene/single-hit phenomenon in humans. Thus, these models perhaps more accurately indicate what overexpression of a single specific gene *can do* rather than what this gene *does* in normal versus malignant tissues. The basic assumption is that, in most cases, these two are the same. Despite the potential limitations inherent in transgenic mouse models, the ability to express an oncogene in the glandular or stromal tissues, or both, of otherwise normal breast tissues provides a unique and powerful technique to address the transforming potential of individual genes.

TABLE 2. *Characteristics of several transgenic models that affect the mammary gland*

Transgene	Promoter	Background	Phenotype	References
c-*myc*	MMTV	FVB	50% of virgin female mice develop tumors in 9–12 mo. Incidence increased and latency reduced with parity. Mice do not lactate well. When crossed with MT-TGF-α, latency was reduced to 66 days in both males and virgin females.	167 164
c-*myc*	WAP	FVB	80% of mice develop tumors after pregnancy (mean latency, 7 mo). Some metastasize to lungs.	151
TGF-α	MT	FVB	64% of multiparous women develop adenomas and adenocarcinomas that are preceded by hypoplastic lesions. Tumors are rare in virgin mice.	148
TGF-α	WAP	FVB	100% of parous mice develop tumors with variable latency. Latency reduced and tumors seen in virgin and male mice when crossed with WAP–c-*myc* mice.	151
TGF-α	MMTV	C57BL/DBA	Females develop hyperplasia throughout the mammary glands. Sporadic tumors in multiparous animals. No abnormalities in males.	196
Middle T	MMTV	FVB	*In situ* carcinoma seen at 3 wk. All male and female mice develop tumors that metastasize to lungs. Metastasis greatly reduced when mice crossed onto plasminogen knockout background.	163 198
ras	MMTV	CD1/C57BL	20% of animals have hyperplasia of the Harderian glands. Sporadic mammary tumors in females and a few males.	199
neu	MMTV	FVB	8-wk-old virgin females have hyperplastic nodules through entire gland. By 89 days 50% of females have tumors and 50% of males by 114 days. Multiple tumors arise synchronously.	200
Mutant p53	WAP	FVB	Mice show normal mammary development, rates of proliferation, and apoptosis. When treated with DMBA, latency of tumor formation is significantly reduced.	166
SV40T	C3(1)	FVB	Mammary hyperplasia seen at 8 wk progressing through a DCIS-like stage to cancer by approximately 16 wk. Homozygous mice lactate poorly.	152

DCIS, ductal carcinoma *in situ*; DMBA, 7,12-dimethylbenz[*a*]anthracene; MMTV, mouse mammary tumor virus; MT, metallothionein; WAP, whey acidic protein.

Table 2 summarizes many of the best-characterized transgenic models of mammary carcinogenesis. It is not intended to provide an exhaustive list or to give a complete description of the models listed. Rather, it is meant to provide some idea of the diversity of mammary phenotypes that have been produced and to give a feel for the classes of transgene that have been used. Further information regarding transgenic models of breast cancer can be found at the Biology of the Mammary Gland web site (http://www.mammary.nih.gov/).

USE OF ANIMAL MODELS FOR OTHER ASPECTS OF BREAST CANCER RESEARCH

These animal models have uses that go beyond studies of biology and therapeutic evaluation. Diet has been widely implicated as a possible contributor to breast cancer risk. The chemically induced models have been most widely applied to these studies and have contributed to considerable controversy in several areas. Perhaps most notable has been the evaluation of dietary fat intake as a risk factor for breast cancer. The DMBA and NMU models have consistently demonstrated sensitivity to the promotional effects of diets high in ω-6 polyunsaturated fatty acids. This effect was further confirmed in two metaanalyses of the numerous animal studies in this field.[168,169] Rose and Connolly[170] have demonstrated the ability of these fatty acids to increase the metastatic potential of human breast cancer xenografts. Data from human studies have been less consistent. Most migrant, international, and case control studies have produced data consistent with the modest effects seen in the animal models.[171,172] Cohort studies have been less consistent, with the majority failing to find such an association. In our rodent studies, we found that *in utero* exposure to the ω-6 polyunsaturated fatty acids can significantly affect breast cancer risk in female offspring.[173] This suggests that the timing of exposure may be critical. In contrast to the potentially promotional effects of the ω-6, there is evidence that the ω-3 polyunsaturated fats may reduce breast cancer risk.[174] Consumption of a diet higher in the ω-3 versus the ω-6 polyunsaturated fatty acids is more common in Eastern than in Western populations.

Other dietary components that have received significant attention include soy and several vitamins and analogues thereof. Soy contains several potentially active components, including the isoflavones (e.g., genistein), Bowman-Birk protease inhibitor, and a relatively beneficial balance of ω-3 to ω-6 fatty acids. The isoflavones have received the most attention. These data suggest that dose and timing are critical. Exposure in xenograft models of postmenopausal breast cancer suggests an estrogenic effect that increases

the risk of breast cancer.[175] A similar effect has been reported for *in utero* exposure.[176] In contrast, exposure in the normal prepubertal gland[177] and exposure in the chemically induced models (probably reflective of relatively early neoplastic disease) can reduce breast cancer risk.[178] Several studies have suggested that the Bowman-Birk protease inhibitor may be the active component against breast and other cancers (reviewed by Kennedy[179]). However, Barnes and associates[180] report that soy retains its chemopreventive activity after autoclaving, a process that was expected to inactivate soy protease inhibitors but not the isoflavones.[180]

Retinoids and analogues of vitamin A continue to attract increasing attention, both as chemopreventive strategies and as new therapeutic modalities for breast cancer. When administered in the diet, both retinyl acetate and *N*-(4-hydroxyphenyl)retinamide reduce chemically induced mammary carcinogenesis.[181] This latter compound is currently under evaluation as a chemopreventive agent for breast cancer in a large study in Italy.

CONCLUSION

Many models for the study of breast cancer are available to the interested investigator. Each has its own advantages and disadvantages, and these should be clearly weighed and evaluated before their use. Because none of the models accurately reflects every aspect of the human disease, some studies may require the use of more than one model.

There are two areas in which more models are needed. Currently, there are relatively few human xenograft models that are ER positive and E2 dependent. Because this represents an early phenotype, the ability to study malignant progression is restricted. There also are few reproducible metastatic models of breast cancer, particularly ER positive and E2 dependent. The MDA-MB-231 and MDA-MB-435 xenografts are ER negative,[182] and the MCF7/FGF4 transfectants are E2 inhibited and tamoxifen stimulated.[183] We described a model derived from a bone metastasis that has a preference for metastasis to bone when inoculated into the heart.[100,101] However, additional metastasis models are required.

Although relatively new in terms of their use in breast cancer research, the polyoma and adenovirus models may provide us with new and important models for breast cancer research. Their further evaluation as models and their similarities and differences to the human disease should be clearly defined. If appropriate, they also could become an important component of our collection of animal models of breast cancer.

ACKNOWLEDGMENT

This work was funded in part by Public Health Service grants R01-CA/AG58022, P30-CA51008, and P50-CA58185.

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Diseases of the Breast, 2nd ed.,
edited by Jay R. Harris.
Lippincott Williams & Wilkins, Philadelphia © 2000.

CHAPTER 23

In Vitro Models

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Breast cancer cells that grow *in vitro* represent one of the most widely used experimental models of breast cancer. For many studies, these models provide the only means to address a specific hypothesis. Most breast cancer cell lines can easily be maintained and studied *in vitro*, and are generally stable with respect to their endocrine responsiveness *in vitro* and *in vivo*. The current understanding of how breast cancer cells respond to estrogenic stimuli is, in no small part, the direct result of *in vitro* studies with human breast cancer cell lines.

Breast cancer cell lines are generally considered in terms of their estrogen-receptor (ER) content—that is, whether they are estrogen receptor–positive (ER+) or estrogen receptor–negative (ER–). This classification largely reflects the clinical value of steroid hormone expression in predicting response to endocrine therapy. However, other characteristics of human breast tumors that tend to follow ER status are frequently exhibited in a similar pattern by cell lines growing *in vitro* and *in vivo*.[1]

A detailed and inclusive review of all breast cancer cell lines and their origins, characteristics, and uses is beyond the scope of this chapter. Consequently, the chapter focuses primarily on the most widely used cell lines, some of their variants, and those models expressing characteristics that closely reflect many of the properties of breast tumors in patients. Also included is a brief description of cell lines that have more unique properties or that are best suited to specific studies, such as endocrine regulation or expression of growth factors or oncogenes. The parental cell lines are readily available (e.g., several are available through the American Type Culture Collection in Rockville, Maryland), and the variants can generally be obtained from their respective originators. For a more detailed description of the characteristics of several of these cell lines, the reader is referred to the review by Engel and Young.[2] Although more than two decades old, this review provides valuable information on the origin and characteristics of 22 breast cancer cell lines. The inclusion of the original citations for almost all of these cell lines provides a valuable reference for the interested reader.

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ESTROGEN-DEPENDENT (ER+/PR+) BREAST CANCER CELL LINES

Approximately 30% of all breast cancer patients respond to endocrine manipulation. The overall response rate to antiestrogens increases to 70% or more in patients whose tumors express both the estrogen receptor (ERs) and progesterone receptor (PR).[3–5] To define the mechanism of action of endocrine therapies and to develop and screen new agents and therapies require models that exhibit an endocrine response profile comparable to that found in breast cancer patients. In this regard, the steroid-dependent breast cancer cell lines have been most useful in studying the growth-inhibitory effects of estrogens, antiestrogens, progestins, and antiprogestins. These cell lines are characterized by a dependence on estrogens for growth *in vitro* or *in vivo* and by sensitivity to the growth-inhibitory effects of antiestrogenic and progestational drugs. In general, steroid-dependent cell lines are poorly invasive and nonmetastatic in athymic nude mice.

MCF-7

The MCF-7 cell line is the most widely used and best characterized of all the human breast cancer cell lines. The mitogenic effects of 17β-estradiol (E2) in human breast cancer cells *in vitro* were initially defined in these cells, as were the *in vitro* inhibitory effects of antiestrogens.[6,7] MCF-7 cells also are growth inhibited by luteinizing hormone–releasing hormone (LHRH) analogues[8] and retinoids.[9–11] The now widely reported E2 dependence for exponential growth both *in vitro* and *in vivo* has provided this cell line with a central role in the study of endocrine responsiveness and malignant progression *in vitro*. Much of the current understanding of the mechanism of action of estrogens and antiestrogens and their role in regulating the proliferation of hormone-dependent breast cancer cells has been derived from work performed using this cellular model. Consequently, a full and detailed discussion of all

the data generated using MCF-7 cells alone is beyond the scope of this chapter.

MCF-7 cells were established from a pleural effusion arising in a postmenopausal woman with breast cancer. The patient had received radiotherapy and endocrine therapy before the appearance of the effusion.[12] In addition to the expression of ER,[12,13] MCF-7 cells express an E2-inducible PR[13,14] and cellular receptors for androgens,[13] LHRH,[8,15] glucocorticoids,[13] insulin,[16] retinoic acid receptors (RAR-α and RAR-γ),[17] and prolactin.[18] The expression/secretion of several growth factors and their receptors also has been described in detail, including the insulinlike growth factors (IGFs),[19–22] type I and type II IGF receptors,[22–24] and several IGF binding proteins[25–27]; transforming growth factor α (TGF-α) and epidermal growth factor receptor (EGFR)[28–30]; several fibroblast growth factors (FGFs)[31] and FGF receptors[32]; and platelet-derived growth factor (PDGF) but not PDGF receptors.[33] The expression of many of these growth factors, and their respective receptors, is strongly E2 regulated in MCF-7 cells.[34] The expression, secretion, and regulation of this wide variety of receptors and ligands has made the MCF-7 cells a valuable model for the study of the role of growth factor and growth factor receptor expression in the proliferation of breast cancer cells.

The E2 dependence for growth, antiestrogen sensitivity, and low metastatic potential of MCF-7 cells has led to the hypothesis that they represent an "early" breast cancer phenotype.[35,36] MCF-7 cells are an excellent model in which to study the process of malignant progression, because they can be subjected to appropriate endocrinologic and physiologic selective pressures for the derivation of variants with more progressed phenotypes. For example, the estrogenic requirement for tumorigenicity in immunocompromised mice has been used to select for E2-independent MCF-7 variants (see below and Chapter 22). Variants have also been selected for antiestrogen resistance, and the extent of induced cross-resistance among structurally diverse antiestrogens has been determined.

T47D

T47D cells were established by Keydar et al. from a 54-year-old patient with an infiltrating ductal carcinoma.[37] The cells express ER, PR, androgen, glucocorticoid,[37] and insulin receptors.[38] Approximately 60% of the original cell line expressed casein.[37,39] The T47D cells are perhaps most notable for their high levels of PR and their remarkable genetic and phenotypic instability.[40–43] Furthermore, these cells exhibit significant growth-regulatory responses to progestational agents.[44,45] Not surprisingly, T47D cells, and several T47D variants that have been obtained, represent the major *in vitro* human breast cancer models for the study of the antiproliferative effects of progestins and antiprogestins and the regulation of PR expression.[42–46] T47D cells also express RAR-α and RAR-γ[17] and are sensitive to the growth-inhibitory effects of retinoids and antiestrogens.[9,10]

The remarkable genetic instability of the T47D cells stands in contrast to the other ER+/PR+ human breast cancer cell lines. Subtle differences in the phenotypic characteristics of all of the ER+/PR+ cell lines are observed from laboratory to laboratory. However, these differences rarely extend to the pattern of expression of steroid hormone receptors, metastatic potential, antiestrogen responsiveness, the estrogen supplementation required for *in vivo* tumorigenicity, or metastatic potential. These are the major phenotypic characteristics of hormone-responsive breast cancer cells.[34–36] In cases in which significant divergent phenotypic responses in other ER+/PR+ cell lines are observed, these divergences are almost always the result of an imposed selective pressure (i.e., *in vivo* or *in vitro* growth in the absence of E2, selection for cytotoxic drug resistance). For example, no bona fide spontaneous ER– or PR– MCF-7 or ZR-75-1 cell lines have been described in detail, other than those generated by the imposition of selective pressures. In marked contrast, simple dilution cloning can produce T47D variants with fundamentally altered endocrine responsiveness, (e.g., ER–/PR+, estrogen unresponsiveness, and antiestrogen resistance).[40–42] Many of these variants are unstable and readily revert to the wild-type phenotype.[40,43] Other T47D variants (e.g., the ER–/PR+ $T47D_{CO}$) have been stable for many years.

ZR-75-1

ZR-75-1 cells were first described by Engel et al. in 1978.[47] They were established from an ascites that developed in a 63-year-old woman with an infiltrating ductal breast carcinoma. This patient had been receiving tamoxifen (tamoxifen citrate) for 3 months before the time when cells were removed to establish the ZR-75-1 cell line.[47] Although the ZR-75-1 cells are ER+/PR+ cells[47,48] and are growth stimulated by estrogens and inhibited by antiestrogens *in vitro*,[47,49] the patient did not respond to tamoxifen.[47] The expression of ER is up-regulated by interferons in these cells, and treatment with interferon 2α can increase sensitivity to tamoxifen.[48] ZR-75-1 cells exhibit an eightfold overexpression of c-*erb*-b2 messenger RNA (mRNA) relative to normal fibroblasts.[50] This overexpression is E2 regulated in ZR-75-1 cells, a down-regulation being associated with E2-induced cell proliferation.[51] Tamoxifen-resistant and hormone-independent ZR-75-1 variants have been described.[49,52,53]

ZR-75-1 cells also express androgen and glucocorticoid receptors[54] and are growth inhibited *in vitro* by progestins[54,55] and somatostatin analogues.[56] They express the RAR-α and RAR-γ retinoic acid isoforms[17] and are growth inhibited by several retinoids.[9,10] ZR-75-1 cells also express low levels of EGFR; altered EGFR expression is associated with tamoxifen resistance and hormone independence.[52,53] The ZR-75-1 cells appear to possess several steroid metabolism pathways.[57–59]

ESTROGEN-INDEPENDENT (ER+/PR+) AND ESTROGEN-RESPONSIVE BREAST CANCER CELL LINES

The estrogenic requirement of the MCF-7, T47D, and ZR-75-1 cells for growth *in vitro* or *in vivo* may not ade-

quately reflect the endocrine environment of many breast tumors in postmenopausal women. Several breast cancer cell lines and variants of the MCF-7, ZR-75-1, and T47D cell lines have been generated that no longer require estrogenic supplementation for growth. These continue to express ER, PR, or both, and many also retain responsiveness to endocrine agents. Several steroid-independent and steroid-responsive cell lines or variants exhibit properties that more closely resemble those of breast tumors in patients than do the steroid-dependent cell lines.

MCF-7/MIII, MCF-7/LCC1: Cells Selected for Estrogen Independence *in Vivo*

Several aspects of the MCF-7 phenotype could be considered potentially inconsistent with the human disease. For example, these cells generally do not proliferate in cell culture media devoid of estrogens and do not form proliferating tumors when orthotopically transplanted into oophorectomized immunodeficient mice. If this dependence on estrogens for growth were to exist in a tumor cell in a postmenopausal woman, the source from which the MCF-7 cells were originally obtained,[12] the cells would not give rise to detectable disease. Despite their apparent metastatic site of origin, MCF-7 cells exhibit few characteristics associated with an invasive/metastatic phenotype. Thus, we have previously suggested that the MCF-7 phenotype represents an early hypothetical breast cancer cell.[35,36]

We wished to determine if, by applying appropriate physiologic and endocrinologic selective pressures, we could obtain cells more representative of many of the ER+/PR+ cells apparent in the breast tumors of postmenopausal women. Thus, we selected MCF-7 cells by xenotransplantation into the mammary fat pads of oophorectomized, athymic, nude mice. After approximately 6 months, we obtained cells (MCF-7/MIII) that were readily reestablished *in vitro*. MCF-7/MIII cells were determined to be of MCF-7 origin by karyotype and isozyme profile analyses.[14] A further selection of MCF-7/MIII cells produced a variant designated MCF-7/LCC1, which exhibits increased metastatic potential and a shorter lag time to tumor appearance compared with MCF-7/MIII.[60,61]

We studied these cells for their respective responses to estrogens and antiestrogens both *in vitro* and *in vivo*. MCF-7/MIII and MCF-7/LCC1 cells proliferate *in vivo* and *in vitro* without estrogen supplementation[14,60] and are responsive to drugs representing each of the major classes of antiestrogens.[60,62] MCF-7/MIII cells also are inhibited by LHRH analogues.[63] Both these variants exhibit an increased metastatic potential *in vivo* and *in vitro*,[14,61] although at a much lower level than ER– cell lines.[1,64] We interpret these observations as indicating that MCF-7/MIII and MCF-7/LCC1 cells exhibit a phenotype representative of many ER+/PR+ cells present in the tumors of postmenopausal breast cancer patients. The phenotype of these cells has been reviewed in detail.[35,36,65]

MCF-7 K3: Cells Selected for Estrogen Independence *in Vitro*

MCF-7 cells also can be selected *in vitro* for their ability to proliferate in the absence of estrogenic stimulation. For example, Katzenellenbogen et al.[66] selected MCF-7 cells in cell culture media devoid of estrogens. The resultant cells (MCF-7 K3) have a phenotype that is generally similar to the MCF-7/MIII cells.[14] In our studies, these cells also form tumors in oophorectomized nude mice, but with a longer doubling time[14] and without an apparently increased metastatic potential (R. Clarke, *unpublished data*, 1992). Other, perhaps more subtle differences appear to exist. For example, the estrogen-induced gene *pS2* is constitutively expressed in MCF-7/LCC1 cells *in vitro* but retains some estrogen-inducible expression *in vivo*.[60] In the MCF-7 K3 cells, pS2 mRNA expression appears to be inhibited by E2.[67] Some evidence exists that MCF-7 K3 cells may be estrogen supersensitive,[67] and estrogen supersensitive MCF-7 cells have been previously reported by others.[68] We have no data for the cells selected *in vivo* (MCF-7/MIII, MCF-7/LCC1) that would clearly suggest that they have a supersensitive phenotype. The biological significance of the apparent differences between cells selected *in vivo* (e.g., MCF-7/MIII) and *in vitro* (e.g., MCF-7 K3) remains to be established.

MCF-7 MKS: Cells Transfected with Fibroblast Growth Factor 4

The FGFs are potent angiogenic growth factors, and several appear to be present in or secreted by human breast cancer cells, or both. Transfection of cells with FGF-4 produces cells (MCF-7 MKS) that are able to generate proliferating tumors in the absence of estrogenic stimulation.[69,70] Whereas MCF-7 cells are generally nonmetastatic, MCF-7 MKS cells produce highly vascular tumors, from which both lymphatic and lung metastases arise with a high frequency. Unlike MCF-7 cells selected for an ability to grow in a low-estrogen environment, MCF-7 MKS cells are stimulated by tamoxifen and inhibited by physiologic concentrations of estrogen.[69] The extent to which this endocrine-inverted phenotype reflects a specific phenotype in the human disease is unclear. However, these cells exhibit an endocrine response pattern similar to that of MCF-7 cells selected for *in vivo* resistance to tamoxifen (see below).

ESTROGEN-INDEPENDENT (ER±/PR±) AND ESTROGEN-UNRESPONSIVE BREAST CANCER CELL LINES

BT 20 (Mutant ER)

The BT 20 cell line is one of the older breast cancer cell lines and was established in 1958 by Lasfargues and Ozzello.[71] The cell line was obtained from a breast cancer patient with an infiltrating ductal carcinoma.[71] BT 20 cells

were initially described as being ER–/PR–,[72] but subsequently ER mRNA was detected.[73] More recently, these cells have been shown to express a novel ER mutant with an exon 5 deletion.[74] This mutation produces a protein that does not bind E2 and would appear ER– by ligand binding. Because some evidence exists that exon 5 mutant ER proteins can be transcriptionally active,[75] BT 20 cells could be considered to be ER+, hormone independent, and hormone unresponsive. These cells also express glucocorticoid receptor (GR),[72] and they have a 16-fold elevation in the mRNA levels of EGFR expression resulting from a fourfold to eightfold amplification.[50,76] BT 20 cells are tumorigenic but nonmetastatic when grown in athymic nude mice.[77]

BT474 (ER–/PR+)

BT474 cells were obtained from a solid primary infiltrating ductal carcinoma of the breast in a 60-year-old woman.[78] The cells express PR but not ER *in vitro*[78] and significantly overexpress c-Erb-b2 due to an amplification in the c-*erb*-b2 gene.[50] The level of c-Erb-b2 mRNA expression in BT474 cells is 128-fold that of normal fibroblasts, whereas EGFR is not overexpressed.[50]

$T47D_{CO}$ (ER–/PR+)

$T47D_{CO}$ cells are a variant of the ER+/PR+ T47D cells[37] and were originally described by Horwitz et al.[79] The most notable feature of these cells is their loss of ER but elevated and constitutive expression of PR.[38,45,79] The cells grow *in vitro* without E2 supplementation and are antiestrogen resistant.[79] Whereas the PR in $T47D_{CO}$ cells is E2 independent, insulin receptor expression is up-regulated by progestins, despite their growth-inhibitory effects.[45] The constitutive expression of PR in the absence of ER makes this an excellent *in vitro* model for screening progestins and antiprogestins, because no complicating requirement for E2 supplementation exists. For example, the antiproliferative effects of the progestin R5020 in T47D cells were initially thought to reflect an antiestrogenic effect.[44] Subsequent data obtained in the $T47D_{CO}$ cells demonstrated that progestins and antiprogestins exert direct growth-inhibitory effects independent of ER-mediated events.[45]

ESTROGEN-UNRESPONSIVE (ER–/PR–) BREAST CANCER CELL LINES

The majority of human breast cancer cell lines are ER–. Just as the ER+ cell lines tend to reflect the nature of ER+ tumors in breast cancer patients, the ER– cell lines exhibit characteristics similar to those of ER– breast tumors. For example, ER– tumors are generally more rapidly growing,[80] more aggressive, and exhibit a poorer prognosis[81–83]; similarly, the ER– cell lines tend to produce rapidly growing tumors in nude mice, several of which are highly invasive and some of which can produce distant metastases.[1,64] None of these cell lines responds to the antiproliferative effects of estrogens and antiestrogens unless exposed to suprapharmacologic doses. However, the absence of response to steroids does not preclude response to other noncytotoxic agents. Several ER– cell lines express retinoic acid receptors[17] and are growth inhibited by retinoids.[11,84]

MDA-MB-231 and MDA-MB-435

The MDA-MB-231 cell line is among the most widely used of the ER– human breast cancer cell lines and is frequently used as a negative control in many laboratories studying the endocrine regulation of breast cancer cell growth. The MDA-MB-231 cells were established from a 51-year-old woman with breast cancer who developed a pleural effusion. The patient had received prior endocrine therapy (oophorectomy) and cytotoxic chemotherapy (initially 5-fluorouracil and then a combination of cyclophosphamide, methotrexate, and adriamycin). She had received the combination regimen 3 weeks before removal of the fluid from which the MDA-MB-231 cell line was isolated.[85] The MDA-MB-231 cells are highly tumorigenic and can produce lung metastases from mammary fat pad tumors in nude mice.[64]

The MDA-MB-435 cells were established from a pleural effusion in a 31-year-old white woman with metastatic breast cancer.[39,85] Unlike many other patients from whom breast cancer cell lines have been obtained, this patient had received no prior systemic therapy.[86] Despite being initially described as nontumorigenic,[87] MDA-MB-435 is generally reported to be highly tumorigenic and is one of the few human breast cancer cell lines that produce lung metastases from solid tumors.[64,88] When growing as xenografts, the growth and metastases of these cells also appear responsive to several dietary manipulations.[89–91] The study of metastasis from the MDA-MB-435 cell line has been greatly simplified by the introduction of a marker (β-galactosidase) that can facilitate visualization of micrometastases.[92]

We have established an ascites variant of the MDA-MB-435 cells (MDA435/LCC6). We have routinely maintained these cells as ascites for several years and assessed their sensitivity to a series of cytotoxic drugs.[86] The ascites has a pattern of responsiveness to single agents that closely reflects the activity of these agents in breast cancer patients. The cells are also easily maintained *in vitro* and can be successfully reestablished as solid tumors or ascites in nude mice. The MDA435/LCC6 cells may provide an alternative to the L1210/P388 murine ascites (leukemia) for the screening of new agents for activity in breast cancer. The MDA435/LCC6 cells also respond to nanomolar concentrations of all-*trans*-retinoic acid, fenretinimide, and 9-*cis*-retinoic acid[11]; this response perhaps reflects the expression of the RAR-α, RAR-β, and RAR-γ isoforms by the parental MDA-MB-435 cells.[17]

Other MDA-MB Cell Lines

Up to 19 cell lines bear the MDA-MB designation; most were derived by Cailleau et al. at the M. D. Anderson Hospital and

Tumor Institute.[39] The basic characteristics and isozyme and karyotype patterns have been previously reported in some detail.[39,93] Most cell lines are ER–, with the notable exception of the MDA-MB-134 and MDA-MB-175 cell lines, which are ER+.[94] Several of these lines are of specific interest. The MDA-MB-468 cells overexpress EGFR[50]; in contrast to other breast cancer cell lines, their growth is inhibited by exogenous EGF.[95] FGF receptors are overexpressed by MDA-MB-175 cells, which are growth inhibited by FGF.[96] The MDA-MB-175 cells also exhibit an eightfold overexpression of c-*erb*-b2 relative to normal fibroblasts.[50] The MDA-MB-361 cells, which were obtained from a brain metastasis,[39] and the MDA-MB-453 cells exhibit a twofold to fourfold amplification of the c-*erb*-b2 gene, overexpressing the gene product by approximately 64-fold.[50] The external domain of c-Erb-b2 is shed from MDA-MB-361 cells and can be detected in the serum of nude mice bearing these xenografts.[97] The MDA-MB-436 cell line was derived from a 43-year-old woman with metastatic breast cancer.[39] These cells are ER– and are sensitive to several cytotoxic drugs. The MDA-MB-436 cells have been used to investigate the effects of insulin and cell-seeding density on methotrexate metabolism[98,99] and the non–ER-mediated effects of estrogens and antiestrogens on both the cytotoxicity of methotrexate[100,101] and cell membrane structure and function.[102]

SkBr3

SkBr3 cells were obtained from a pleural effusion that developed in a 43-year-old patient with a breast adenocarcinoma.[103] These cells have been widely used in the study of c-Erb-b2 expression, because they overexpress c-Erb-b2 128-fold relative to normal fibroblasts owing to a fourfold to eightfold amplification of this gene.[50] SkBr3 cells also secrete a truncated c-Erb-b2 into their cell culture medium.[104] The coexpression of EGFR and c-Erb-b2 has enabled studies into the mechanisms of EGF-induced heterodimerization.[105]

NEW BREAST CANCER CELL LINES

The following section is not intended to be exhaustive but includes several new cell lines with some novel or unusual characteristics. The methods of isolation vary across studies, but all cell lines have been confirmed to be of breast origin. When the information is available, tumorigenicity, oncogene expression, and other potentially relevant information is described.

The establishment of new cell lines is a difficult and time-consuming process. Primary cultures can be initiated with a relatively high success rate.[106,107] Biopsy material can be cultured in a manner that allows for the preferential growth of neoplastic rather than normal epithelial cells.[108] However, the proportion of these cultures that spontaneously develop into established cell lines—for example, lines that can be maintained successfully for over 50 passages—is relatively low. In a study of 135 primary breast cancers, only 10 produced cell lines. All were negative for ER.[109] The success rate appears similar when the material for culture is derived from lymph node metastases. Thus, tumor stage would not seem to be a particularly good predictor for *in vitro* establishment.[106] The very poor success rate in establishing ER+ cell lines remains problematic.

Although ER can be overexpressed in ER– breast cancer cells by transfection, the resulting cells are almost exclusively growth inhibited by physiologic concentrations of estrogens.[110] A similar phenotype is reported for overexpression of ER in the normal human breast epithelial cell line MCF10A,[111] although the antiproliferative responses to estrogens appear relatively small. Breast tumors generally contain these levels of intratumor estrogens, regardless of the patient's menopausal status.[112] Such tumors might not arise if these intratumor estrogens were growth inhibitory, or at least would not arise until this signaling was eliminated or overcome. Furthermore, the administration of exogenous estrogens—for example, estrogen-based hormone replacement therapy—generally increases breast cancer risk.[113] This is consistent with the mitogenic effects of estrogens in cells that "normally" express ER, such as MCF-7 cells, and the growth-inhibitory effects of antiestrogens. Thus, the phenotypic relevance of these transfected cells is difficult to determine. Some transfectants can regulate estrogen-responsive element (ERE)–regulated gene expression in transient transfection assays but do not regulate endogenous genes, such as the genes for PR, pS2, and cathepsin D, in response to estrogens.[111] Thus, the function of some estrogen-regulated genes may be unaffected by expression of ER.

Some breast cancers possibly may arise from cells that were normally negative for ER,[114] and cellular signaling within these populations may be different from that in cells that normally express ER and require estrogens for proliferation. Some critical genes may be regulated in a direction not seen in cells that normally express ER, perhaps accounting for the inverted—that is, estrogen-inhibited—phenotype. If the ER function is significantly different than that in cells which normally express ER, the use of these models to study signaling to estrogenic/antiestrogenic responses may require careful consideration.

One cell population has been studied that is naturally inhibited by estrogens—namely, vascular smooth muscle cells.[115–117] These cells are naturally growth inhibited by estrogens, a finding that is not surprising, as estrogen is known to reduce the risk of cardiovascular disease. One mechanism might be through the inhibition of vascular smooth muscle cell proliferation after vascular injury.[117] Although an estrogen-inhibited phenotype is not physiologically unimportant in other tissues, the extent to which it applies to breast cancer is unclear.

BRC-230

The patient from whom cells for this line were obtained was a 79-year-old woman with a metastatic, infiltrating ductal car-

cinoma. The primary tumor was ER–/PR– and exhibited a high thymidine (TdR)-labeling index.[118] The cell line, designated BRC-230, was established from surgical material obtained from the primary tumor. The cells are ER–/PR– and show no evidence of amplification or rearrangement of c-*erb*-b2, c-*myc,* or *mdr*-1. BRC-30 cells are tumorigenic in nude mice and produce carcinoembryonic antigen, CA15-3, CA19-9, and CA125.[118] Of potential interest is the pattern of chemosensitivity, which closely reflects that seen in the patient. BRC-230 cells exhibit a multiple drug–resistant phenotype and are resistant to doxorubicin hydrochloride, etoposide, idarubicin hydrochloride, mitoxantrone hydrochloride, 4'-epidoxorubicin, and 4-idroperoxy-cyclophosphamide.[118] BRC-20 cells may be useful in studying non–*MDR1*-mediated multiple drug resistance.

HMT-3909S1 and HMT-3909S8

Petersen et al.[119] have described two cell lines derived from the same primary tumor. The primary tumor was an infiltrating ductal carcinoma that arose in a 61-year-old white woman. The patient had received no prior therapy. The cell lines were established in serum-free media.[119] HMT-3909 is nontumorigenic, whereas the HMT-3909S8 line forms tumors in athymic nude mice. HMT-3909S8 cells are aneuploid and express the mesenchymal glycoprotein vimentin, keratins 8 and 18, and the MAM-6 glycoprotein. HMT-3909S1 cells express vimentin and several keratins, including keratin 18, but MAM-6 reactivity is weak and keratin 8 staining is not detected.[119] The cells are not entirely ER–, but expression appears very low. The presence of two phenotypically distinct cell lines, each derived from a single hypothetical stem cell, may provide a novel means to study progression and acquisition of phenotypic diversity in tumors.

KPL-3C

KPL-3C cells were derived from a 37-year-old Japanese woman with invasive ductal carcinoma. The patient was initially treated by radical mastectomy and subsequently received radiotherapy to the locally recurrent lesions and systemic chemoendocrine therapy. Liver metastasis and a pleural effusion were subsequently diagnosed. The cell line was established from the pleural fluid.[120] The resulting cell line (KPL-3C) is tumorigenic in athymic nude mice, with a doubling time of approximately 7 days, and produces tumors reminiscent of comedo-type intraductal neoplasms. The tumors often exhibit an area of central necrosis characterized by microcalcification. The cells express cytokeratin, epithelial membrane antigen, carcinoembryonic antigen, and CA15-3, but do not express either vimentin or c-Erb-b2. Expression of ER and PR is low, with the levels reported as approximately 15 fmol per mg protein and 14 fmol per mg protein, respectively.[120] The cell population doubling time *in vitro* is approximately 72 hours. A potentially unusual characteristic of this cell line is its secretion of parathyroid hormone–related protein. Interestingly, the patient from whom these cells were derived required treatment for humoral hypercalcemia. This cell line may be useful for studying the role of tumor-derived parathyroid hormone–related protein in humoral hypercalcemia of malignancy.[120]

LCC15-MB

The LCC15-MB cell line was established from a femoral bone metastasis. The patient was a 29-year-old woman initially diagnosed with an infiltrating ductal mammary adenocarcinoma.[121] Approximately 3 years after the initial primary tumor was diagnosed, the patient presented with acute bone metastasis. Material from the bone metastasis, a poorly differentiated adenocarcinoma lacking ER, PR, and *erb*-b2 expression, was used to establish the cell culture. The LCC15-MB cells exhibit these characteristics, although ER can be reexpressed by treatment of the LCC15-MB cells for 5 days with 5-aza-2'-deoxycytidine. LCC15-MB cells are tumorigenic in athymic nude mice and produce long-bone metastases after intracardiac injection.[121] These cells strongly express vimentin. The presence of keratin 18 mRNA has been demonstrated using assay by polymerase chain reaction, but the low overall levels of keratin protein and the lack of keratin 19 mRNA suggest that these cells have selectively lost epithelial characteristics while gaining a more mesenchymal phenotype, consistent with the epithelial to mesenchymal transition that can occur during malignant progression in breast cancer.[121] The cells are invasive in the *in vitro* Boyden chamber assay and activate matrix metalloprotease 2 after treatment with concanavalin A.[122] Significantly, LCC15-MB cells express the bone matrix protein osteopontin, and this expression is retained by subcutaneous xenografts and intraosseous metastases.[122] The LCC15-MB cells provide a unique model in which to study bone metastasis and the role of osteopontin in this process.

MFM-233

The MFM-233 cell line was derived from a pleural effusion that arose in a postmenopausal patient. The patient had not received any prior treatment and presented with a widespread grade 3 ductal carcinoma. The established cell line was designated MFM-223.[123] These cells are tumorigenic in nude mice, producing moderate to poorly differentiated adenocarcinomas. MFM-233 cells express cytokeratins 8 and 18, epithelial membrane antigen, and milk fat globulins 1 and 2. Relatively low levels of ER (5 to 10 fmol/mg protein) are expressed, but PR is not detected in the cultures and is not induced by estradiol treatment. In contrast, expression of androgen receptors is high (160 fmol/mg protein), and proliferation of the cells is inhibited by more than 0.01 nanomolar dihydrotestosterone.[123] This cell line may prove useful in the study of androgen responsiveness and signaling.

MODELS OF ACQUIRED ANTIESTROGEN RESISTANCE (ER+ CELLS)

Although acquired resistance to antiestrogens is one of the more pressing clinical problems in breast cancer, few *in vitro* models exist for the analysis of this aspect of malignant progression. The most common approaches to the isolation of antiestrogen-resistant cells use an *in vitro* selection of hormone-dependent cells against either a high single dose of antiestrogen[124] or a stepwise increase in concentrations of drug. These approaches have been widely used to generate variants of cell lines resistant to many antineoplastic agents. Several problems are encountered when this approach is applied to generate antiestrogen-resistant variants of estrogen-dependent breast cancer cells. For example, isolation of resistant clones that retain stability for several years has often been difficult. Several laboratories have reported resistant variants that revert to a sensitive phenotype with a high frequency.[49,125–127] Some cell lines alter other critical aspects of their phenotypes. The MCF-7 variant LY-2 has become nontumorigenic.[62] MCF-7 cells selected *in vivo* can become dependent on or stimulated by tamoxifen.[128,129] Cells transfected with FGFs also reverse their endocrine responsiveness, becoming stimulated by tamoxifen and inhibited by physiologic concentrations of estrogens.[70] These models may reflect a tamoxifen-withdrawal effect, although the prevalence of this response in humans is difficult to determine.

LY-2: MCF-7 Cells Selected against a Benzothiophene (LY117018) *in Vitro*

The MCF-7 variant LY-2[124] is perhaps the most stable antiestrogen-resistant variant and was generated in 1985. These cells were selected *in vitro* in an anchorage-independent (soft agar) colony assay for resistance against LY117018.[124] LY-2 cells have been demonstrated to be cross-resistant to drugs representative of the major structural classes of antiestrogens, including nafoxidine, 4-hydroxytamoxifen, and ICI 164,384.[62] In addition to exhibiting a significant shift in their dose-response relationship for antiestrogens, LY-2 cells exhibit a blunted mitogenic response to E2.[124] However, LY-2 cells have lost their ability to form proliferating tumors in oophorectomized or E2-supplemented nude mice,[62] limiting their use to *in vitro* studies.

Although the precise resistance mechanism in the LY-2 cells is unclear, these cells express ER levels approximately one-third those of their parental MCF-7 cells and have become negative for PR.[124] A reduced level of ER expression would be expected to induce resistance to all antiestrogens, because interaction with ER is likely to be the most important early event in antiestrogen function. Thus, the altered ER levels or the reduced ability to mount an estrogenic response in the LY-2 cells may explain their antiestrogen-resistance pattern. LY-2 cells still express levels of ER that would be considered high in a breast tumor biopsy.[124] Furthermore, the remaining ER appears normal—that is, it is not mutated or altered.[130] The LY-2 cells may mimic some aspects of the antiestrogen-resistance profile in patients with ER+/PR– tumors.

MCF-7 Cells Selected against Tamoxifen *in Vivo*

Several groups have generated resistance models by selecting MCF-7 xenografts growing in nude mice against tamoxifen, an approach that would appear to more closely mimic the human disease than *in vitro* selection. However, MCF-7 cells do not form proliferating tumors in castrated female mice, which have an endocrine environment similar to that of postmenopausal women.[131–133] Because the xenografts would not be proliferating, their growth could not be further suppressed; tamoxifen is generally considered a cytostatic, not cytotoxic, drug. Consequently, it might be predicted that the most efficient response to such a selective pressure would be a change in the cell's perception of tamoxifen from inhibition to the widely documented partial agonist (growth promotion at low concentrations) properties of tamoxifen.[134] Indeed, the resultant tumors exhibit a tamoxifen-stimulated/tamoxifen-dependent phenotype,[128,129] suggesting that this "inverted" phenotype reflects a sensitization to the partial agonist (estrogenic) effects of the triphenylethylenes.[134] Tamoxifen dependence is evidenced by withdrawal responses to tamoxifen that induce regression of the xenografts.[128] More recently, an ER variant has been identified that may explain these changes in responses to antiestrogens.[135,136] Jiang et al. have identified a glycine-to-valine mutation at amino acid position 400 in the ER protein. When transfected into breast cancer cells, this mutation confers a growth-inhibitory response to estrogens and a growth-stimulatory response to antiestrogens.[135,136]

A breast tumor in a patient that possessed a tamoxifen-dependent phenotype could respond to removal of tamoxifen by exhibiting a tamoxifen-withdrawal response. Withdrawal responses have been widely reported for other endocrine therapies, including high-dose estrogen and progestin treatment.[137] Whether this occurs for antiestrogens is unclear, because the incidence of tamoxifen withdrawal responses has not been clearly defined and documented. Several anecdotal and single case reports of tamoxifen withdrawal responses have been published.[138–143] Several larger studies indicate a low incidence of tamoxifen withdrawal responses.[144,145] Thus, the data from these models may be predicting a response yet to be clearly demonstrated in the clinic or demonstrating an experimental artifact. Should these models be correct, the potential for a significant incidence of tamoxifen withdrawal responses could indicate an important, and potentially underestimated, clinical response pattern.

Selection of Hormone-Independent but Hormone-Responsive Cells against 4-Hydroxytamoxifen

Rather than use hormone-dependent cells and risk a loss of tumorigenicity (e.g., the LY-2 phenotype) or select *in vivo* using hormone-dependent cells and obtain a tamoxifen-stimulated phenotype, we hypothesized that cells already hormone independent and responsive might provide a more appropriate starting point for the generation of resistant variants. These cells already proliferate in the absence of estrogenic stimulation both *in vivo* and *in vitro*. To eliminate species-specific metabolic differences between rodents and humans, we chose to perform a stepwise selection of the MCF-7/LCC1 cells *in vitro* against the potent tamoxifen metabolite 4-hydroxytamoxifen. We obtained a stable resistant population designated MCF-7/LCC2. These cells are resistant to tamoxifen when growing either *in vitro* or as xenografts in nude mice[146] and have remained stably resistant in the absence of selective pressure.

We determined the likely cross-resistance profile of these cells by assessing their *in vitro* growth response to steroidal antiestrogens. Although resistant to tamoxifen, MCF-7/LCC2 cells are not cross-resistant to either ICI 182,780 or ICI 164,384.[146,147] This response pattern suggested that some patients who initially respond to tamoxifen but ultimately relapse may retain the ability to respond to a steroidal antiestrogen. Subsequently, this resistance pattern has been observed in preliminary data from a Phase I trial of ICI 182,780 in heavily tamoxifen pretreated patients.[148] These data indicate that, as predicted by the MCF-7/LCC2 phenotype, patients that ultimately relapse on tamoxifen can obtain responses to a subsequent steroidal antiestrogen treatment. Thus, an *in vitro* observation correctly predicted a subsequent pattern of response in breast cancer patients. These data suggest that the clinical responses to ICI 182,780 probably represent a genuine direct antitumor effect, rather than a possible tamoxifen withdrawal response, and suggest that the MCF-7/LCC2 phenotype is not merely an *in vitro* artifact. The relevance of these cells and their phenotypes has been reviewed.[36,65]

Cells Selected for Resistance to Steroidal Antiestrogens

MCF-7/LCC1 cells selected against the steroidal antiestrogen ICI 182,780 have been isolated and characterized. An *in vitro* stepwise selection was used similar to that used to generate the MCF-7/LCC2 cells. The stable ICI 182,780–resistant population was designated MCF-7/LCC9. MCF-7/LCC9 cells are resistant to ICI 182,780 *in vitro* and *in vivo*.[149] Our data indicate that these cells exhibit cross-resistance to tamoxifen, even though the cells have not been exposed to a triphenylethylene antiestrogen. If correct, this pattern of *in vitro* resistance would suggest that patients may be better served if treated initially with tamoxifen and subsequently with a steroidal antiestrogen, rather than vice versa. The validity of this prediction remains to be tested in patients.

MODELS FOR STUDYING MULTIPLE-DRUG RESISTANCE (*MDR1*/GP170)

Many breast tumors are often initially responsive to cytotoxic chemotherapy. Almost all develop a multiple drug–resistant phenotype, however, and this is ultimately responsible for the failure of current cytotoxic regimens.[34] Acquired resistance is frequently associated with expression of the *MDR1* gene and its gp170 glycoprotein product. The level and incidence of detectable *MDR1*/gp170 expression is significantly higher in the tumors of treated versus untreated breast cancer patients[150–152] and correlates with *in vitro* resistance to cytotoxic drugs.[152–154] Several *in vitro* models have been established with which to screen for new agents that can reverse this form of multiple drug resistance.

Cells Selected for Resistance against Adriamycin

Cell lines selected *in vitro* for resistance to adriamycin frequently overexpress gp170, often as a result of amplification of the *MDR1* gene. Among the most widely used cell lines are the MCF-7^{ADR} line[155] and the HeLa (ovarian carcinoma) variant KbV series.[156] The origin of the MCF-7^{ADR} cells, as used in the current National Institutes of Health (NIH) drug-screening program, has been questioned. The MCF-7 origin of these cells could not readily be determined, and the cell line has been redesignated NCI/ADR-RES.[157] The extent to which these cells can be used as a specific model of multiple drug–resistant breast cancer is unclear.

One problem with cells selected *in vitro* is that they frequently acquire multiple drug–resistance mechanisms. For example, we have demonstrated that NCI/ADR-RES (MCF-7^{ADR}) cells, but not *MDR1*-transduced MCF-7 cells (CL 10.3), are cross-resistant to tumor necrosis factor.[158] Because both adriamycin and tumor necrosis factor can inhibit cells by the generation of free radicals,[159,160] this cross-resistance in NCI/ADR-RES cells strongly suggests the presence of adriamycin-resistant mechanisms in addition to gp170, including altered expression of manganous superoxide dismutase.[158] Indeed, NCI/ADR-RES cells also exhibit increased glutathione transferase and topoisomerase II activities[161,162] and have become estrogen independent and antiestrogen resistant due to their loss of steroid hormone receptor expression.[155]

The complexity of the resistance phenotype in these cells may explain why the gp170-reversing potency of isomers of flupenthixol identified in NCI/ADR-RES (MCF-7^{ADR}) cells could not be confirmed in *MDR1*-transfected NIH 3T3 cells,[163] which suggests a non–gp170-mediated mechanism. Although NCI/ADR-RES cells are clearly of considerable use for screening new resistance-modifying agents and combinations, their use for detailed mechanistic studies of resistance reversal may be limited. These cells are widely used and very well characterized, however, and they provide an important benchmark for comparing data among different studies.

Cells Transduced with the *MDR1* Gene

To obtain cells in cases in which gp170 is the major multiple drug–resistance mechanism, a cloned, E2-dependent, MCF-7 human breast cancer subline was transduced with a retroviral vector directing the constitutive expression of the *MDR1* gene.[164] After selection in the presence of the gp170 substrate colchicine, cell populations (MCF-7^{MDR1}) were isolated, and their ability to produce gp170 was determined by radioimmunoprecipitation.[164] In this study, one of the MCF-7^{MDR1} clones designated CL 10.3 was used. Transduced cells express high levels of a 170-kd glycoprotein exhibiting immunoreactivity with specific anti-gp170 antibodies. Immunoreactivity is not detected in either the parental MCF-7 cells or MCF-7 cells transduced with a control pSV2neo vector. The level of expression of MCF-7^{MDR1} cells is estimated to be within twofold to threefold of that expressed by the adriamycin-selected NCI/ADR-RES cells.[163] The function of the expressed glycoprotein was confirmed by determining the sensitivity of parental and MCF-7^{MDR1} cells to a gp170 substrate (adriamycin) and to a non-gp170 substrate (gossypol).[164,165] Transduced cells have a tenfold greater IC_{50} for adriamycin, whereas sensitivity to gossypol is equivalent in both parental and transduced cells.[164] A similar relationship has been observed for colchicine and the non-gp170 substrate methotrexate (R. Clarke and F. Leonessa, *unpublished observations*, 1990).

The increase in resistance exhibited by the transduced MCF-7^{MDR1} cells would be expected to be sufficient to induce clinical resistance in tumors. Perturbations in energy metabolism in the MCF-7^{MDR1} cells have also been observed that are not present in the parental cells.[165] *MDR1*-transduced cells retain ER and PR expression and sensitivity to the triphenylethylene antiestrogen 4-hydroxytamoxifen.[164] Expression of the estrogen-inducible pS2 and EGFR genes are similar in parental and MCF-7^{MDR1} cells.[164] EGFR is upregulated, and pS2 expression is lost in NCI/ADR-RES cells.[155,164] The data indicate that overexpression of the *MDR1* gene alone confers a multiple drug–resistance phenotype but does not result in either cross-resistance to antiestrogens or a loss of steroid hormone receptor expression.[164]

The ascites variant MDA435/LCC6 of the ER– breast cancer cell line MDA-MB-435 has been transduced with the *MDR1* complementary DNA. The MDA435/LCC6 cells appear to retain the major characteristics of their parental cells; that is, they are ER–, highly tumorigenic, invasive, and metastatic.[166] When the cells are grown as an ascites, the mice become moribund within a reproducible time (approximately 30 days) and exhibit a pattern of responses to established cytotoxic drugs that closely reflects the activity of the agents when administered as single agents to previously untreated breast cancer patients.[166] The *MDR1*-transduced cells (MDA435/LCC6^{MDR1}) provide an ER– model for comparison with the ER+ MCF-7^{MDR1} CL 10.3 cells. The ascites variants provide an alternative to the murine leukemia ascites models (e.g., L1210, P388) for screening gp170-reversing agents.[166]

NORMAL BREAST EPITHELIAL CELLS AND THEIR DERIVATIVES

Culturing Normal Breast Epithelial Cells *in Vitro*

Major advances have been made in the culture of normal mammary cells from both humans and rodents. Not all of the approaches can be discussed in detail here, but several excellent reviews are available.[167,168] Of particular importance are models that allow for the coculturing of stromal and epithelial cells, because the interactions among these populations appear critical for normal glandular development and function. The approaches reviewed by Ip and Darcy[167] demonstrate the ability of cells maintained *in vitro* to complete a phenotypically normal lobuloalveolar development. These structures secrete milk proteins in response to appropriate hormonal stimuli and undergo an apparent involution on hormone withdrawal.[167] The culture techniques have been optimized for, and widely applied to, both human and rodent mammary cells.[167,168] For example, normal human epithelial cells proliferate and differentiate in a three-dimensional sponge-gel matrix culture system.[169]

These approaches require the isolation of viable epithelial or stromal cells from solid tissues. Many investigators appear to use one of several collagenase-based disaggregation methods,[167,170] but explant, organ culture, and organoid approaches also are successful.[106,167,171] The most effective approaches generally differ from the standard cell culture techniques used to propagate and study breast cancer cells, primarily in the provision of a three dimensional environment and the inclusion of stromal cells.[167,168] The success rate in establishing primary cultures of both normal and neoplastic mammary tissues has increased significantly. Even relatively simple approaches can produce short-term cultures on plastic with good reproducibility. For example, Volpi et al. have reported success rates of 83% for primary human breast cancers and 78% for normal breast tissue.[106]

Several specialized cell culture media have been generated that have greatly increased the success rate for establishing primary cultures. In general, these are semi-synthetic media that contain little or no serum, have low levels of Ca^{2+}, and are supplemented with various hormones, growth factors, and chemically undefined ingredients, such as conditioned cell culture media and bovine pituitary extract.[106,172–175]

Although these cells represent primary cultures—that is, they have a finite life span *in vitro*—they may be immortalized by treating them with carcinogens and transformed by inducing an overexpression of several oncogenes. As with the neoplastic breast cell lines, several caveats should be borne in mind. For example, the primary and immortalized cells are adapted to *in vitro* growth, and some of their

expressed (or repressed) characteristics may be more closely associated with this adaptation than their normal *in vivo* function. However, this concern most likely is minimized when three-dimensional culture matrices are used and stromal cells are included. Immortalized cells are continually proliferating, a state quite different from the resting tissues from which their parental cells were derived.

All the available cell lines established from normal mammary cells are ER–. ER has been introduced into several normal human breast epithelial cell lines. However, the resultant phenotype is growth inhibited by estradiol.[176] A similar phenotype occurs when breast cancer cells are transfected with ER.[110] This E2-inhibited phenotype appears counterintuitive, because estrogens are generally considered mitogens in both normal and neoplastic breast tissues. Nevertheless, these potentially "normal" cells are important models that provide the opportunity to study aspects of the biology of normal mammary epithelial cells, to identify agents that may contribute to the malignant transformation of normal mammary cells, and to determine phenotypic and genotypic perturbations associated with this process.

Benzo(*a*)Pyrene-Immortalized 184 and B5 Lineages from Reduction Mammoplasties

Stampfer and Bartley[173] have successfully established primary organoid cultures from normal reduction mammoplasty tissues. The source tissues for these cultures were essentially resting, in that they were not obtained during a functional or active period, such as early pregnancy, lactation, or involution.[173] These cells can readily be immortalized by treatment with benzo(*a*)pyrene (e.g., 184 cells). Immortalized normal mammary epithelial cells can exhibit evidence of their breast epithelial origin. For example, the cells are clearly epithelial,[172] express several human milk fat globulin antigens, and synthesize α-lactalbumin and β-casein.[173] Although immortalized—that is, they can be maintained continuously *in vitro*—these cells are not considered transformed according to several criteria, including their inability to form tumors in nude mice or significant anchorage-independent growth.[173]

Transformation of Immortalized Human Mammary Epithelial Cells with Oncogenes

The introduction of viral or cellular oncogenes into benzo(*a*)pyrene-immortalized human mammary epithelial cell lines results in a stepwise progression from a normal to a malignant phenotype.[173,177–179] Two distinct immortalized lineages (184AIN4 and 184B5) have been characterized after transformation by several viral oncogenes. Tumorigenicity in nude mice is observed after infection of the benzo(*a*)pyrene-immortalized 184A1N4 subline with v-Ha-*ras* (A1N4-H), but not v-*mos* (A1N4-M), c-*myc* (A1N4-myc), or *SV40T* (A1N4-T; after limited passaging of cells).[177] Although they are nontumorigenic, v-*mos*-, c-*myc*-, and *SV40T*-transformed cells do exhibit phenotypic transformation and autonomy from growth factors *in vitro* to varying degrees. Combination of v-Ha-*ras* with v-*mos* (A1N4-MH) or *SV40T* (A1N4-TH) resulted in highly malignant and metastatic tumors in the nude mouse.[177,178] Consistent with the effects of v-Ha-*ras* on the A1N4 cell line, infection of the 184B5 subline with v-Ki-*ras* (B5kTu cells) also confers tumorigenicity in nude mice.[173]

MCF-10A, MCF10AT, and MCF10AT1

Soule et al.[175] have described a spontaneously immortalized "normal" human breast epithelial cell line (MCF-10). The cells were isolated from mastectomy tissue obtained from a 36-year-old premenopausal woman with benign fibrocystic disease. After 849 days in culture, a population designated MCF-10A was established. These cells exhibit a stable t(3;9)(3p13;9p22) translocation.[175] The MCF-10A cells resemble luminal epithelial cells rather than myoepithelial cells, and express antigens for several keratins and epithelial sialomucins.[180] The cells are nontumorigenic in nude mice and do not exhibit anchorage-independent growth.[175] These cells have also been used to assess the transforming ability of several oncogenes. Transfection with the ER gene was not sufficient to produce transformation.[111] MCF-10 cells cotransfected with the *erb*-b2 and Ha-*ras* oncogenes (MCF-10A HE) exhibited a substantial increase in soft agar clonogenicity but lacked significant tumorigenicity in nude mice.[181]

Transformation with the Ha-*ras* oncogene alone (MCF10AT) caused an increase in clonogenicity chemotaxis and degradation of basement membrane *in vitro*.[182] However, the cells are poorly tumorigenic in nude mice. Small, palpable nodules do arise, and these can persist.[183] Sporadic progression to carcinoma was observed, and cells from one of these was reestablished *in vitro* (MCF10AT1). MCF10T1 cells can produce simple ducts when embedded in Matrigel and transplanted into immunodeficient mice.[183,184] Up to 25% progress to invasive carcinoma.[184] Expression of c-*erb*-b2 was detected in 50% of the atypical hyperplasias and 78% of the invasive adenocarcinomas.[185] These cells provide an important and unique model for the progression from atypical hyperplasia to carcinoma.

HBL-100

The HBL-100 cell line is comprised of cells obtained from an early lactation sample.[186] The cells have been described as being of myoepithelial origin.[187] The donor was an apparently healthy woman and had no evidence of breast lesions.[186,188] The cells can form colonies in soft agar, however, and are aneuploid.[186] Although early passage cells are nontumorigenic, HBL-100 cells become tumorigenic after repeated passage *in vitro*, generally around passage 70.[187,189–191] This appears to be associated with the acquisition of specific marker chromosomes[192] and alterations in microfilament and microtubules,[189] and overexpression of

an 89-kd heat shock protein in late passage.[193] Although some HBL-100 cell stocks contain Mason-Pfizer monkey virus,[194] the ability to acquire a transformed phenotype appears to be related to the incorporation of *SV40* sequences into the genome.[188,190,195,196] The cells bind and respond to glucocorticoids and EGF,[197] and express functional β-adrenergic receptors[198] and the IGF-I receptor.[199] Although HBL-100 cells are negative for ER and prolactin receptors,[200] they may require activity of their FGF-2 autocrine loop for maximal proliferation.[201]

Although these cells are derived from an apparently normal donor, it is not entirely clear that they can be considered to represent normal mammary cells. Care must be exercised when selecting HBL-100 cells as a model of normal breast cells. For such a model, their use should probably be restricted to cultures of as early a passage as possible, and almost certainly to cells of passages earlier than 70. Under other circumstances, HBL-100 cells provide a potentially useful model in which to study transformation and progression.

IN VITRO MODELS FOR STUDYING INVASION AND METASTASIS

To metastasize effectively, cells must accomplish a complex compendium of activities, including escape from the primary lesion, avoidance of immune surveillance, and penetration into normal tissue at distant sites.[202] Invasion of extracellular matrices occurs repeatedly in this process, and basement membrane invasion, in particular, has received considerable attention.[203] The loss of basement membrane at the parenchymal-mesenchymal interface of locally invasive tumors has been closely associated with metastatic dissemination.[204–206] The uniformity of basement membrane composition and structure suggests that the molecular mechanisms involved in basement membrane recognition, attachment, degradation, and traversal may yield novel targets for cancer therapy. Several *in vitro* models have been used to study the process of basement membrane invasion and its relationship to malignant progression.

In Vitro Assays for Invasive Potential

The development of Matrigel, a reconstituted basement membrane extract from the EHS (Engelbreth-Holm-Swarm) sarcoma,[207] has been instrumental in facilitating compositional and functional analyses of basement membranes. Matrigel is liquid at 4°C, so that various manipulations are possible before it sets into a homogeneous gel at 37°C. Matrigel contains the major basement membrane components, including laminin, collagen type IV, and heparan sulfate proteoglycan. Matrigel has been used in two different assays to examine *in vitro* invasiveness of breast cancer cells.[208]

The Boyden chamber chemoinvasion assay[209] compares the ability of cells to traverse a Matrigel-coated polycarbonate filter as they migrate toward different chemical attractants. Invasive cells, stained on the lower filter surface, can be quantitated either by image analysis or crystal violet staining.[210] Although the assay was originally developed in modified blind-well Boyden chambers, two-compartment chamber systems, such as Transwell from Gibco (Rockville, MD) and Bio-Coat wells from Collaborative Research/Beckton Dickinson Labware (Franklin Lakes, NJ), have been used successfully. Other adaptations include prelabeling of the cells with either a nontoxic fluorescent dye or radioactive agent to facilitate quantitation of invaded cells.

The ability of cells to form invasive colonies when embedded in a three-dimensional gel of Matrigel is compared qualitatively in the Matrigel-outgrowth assay.[209,210] Cells dispersed in a three-dimensional layer of Matrigel are examined after culture for 2 to 10 days. Although dispersal of single cells throughout the upper layer of Matrigel provides the most stringent test for invasive outgrowth, characteristic morphologies can be achieved more rapidly with cells sandwiched between two layers of Matrigel or simply plated on top of Matrigel.

The presence or lack of ER is an important determinant of both prognosis and choice of treatment of breast cancer. The hormone-responsive or hormone-dependent breast cancer cell lines represent a model system for the analysis of hormonal influences on the invasive process. Effects of estrogens, antiestrogens, and progestins on the *in vitro* invasiveness of steroid-dependent and steroid-responsive human breast cancer cells have been reviewed.[211–214] In addition, progression to hormone independence has implications for invasiveness and metastasis. For example, MCF-7/MIII and MCF-7/LCC1 cells have been shown to acquire an increased metastatic potential as they become estrogen independent.[61] This increased metastatic potential is reflected in increased activity in the Boyden chamber[62] (but not in Matrigel outgrowth assays), increased local invasiveness *in vivo*, and an ability to produce occasional distant metastases in nude mice.[61]

To examine the hypothesis that ER– human breast cancer cell lines are constitutively more invasive than their ER+ counterparts, a large number of the human breast cancer cell lines described above have been examined for invasiveness in the Boyden chamber assay *in vitro* and for metastatic potential in the nude mouse. These studies clearly indicate that the majority of ER– cell lines are inherently more aggressive than ER+ cells both *in vitro* and *in vivo*.[1,208] Because the incidence of distant metastases is significantly lower and less reproducible than that observed in the MDA-MB-435 cells, the MCF-7/MIII and MCF-7/LCC1 cells appear to represent a phenotype intermediate between the poorly invasive MCF-7 and the metastatic MDA-MB-435.[61]

Cell Lines and the Epithelial-to-Mesenchymal Transition

An emerging development in progression studies for breast carcinoma is the immunocytochemical analysis of markers characteristic of epithelial or mesenchymal phenotypes. The

mesenchymal intermediate filament glycoprotein vimentin (VIM) has been associated with lack of ER, high growth fraction, and poor nuclear grade in human breast cancer.[215–219] VIM expression in the tumor component is indicative of an epithelial-to-mesenchymal transition, which may occur during the process of malignant progression. Consistent with this notion, the epithelial marker E-cadherin, a homotypic cell adhesion molecule, is lost from more aggressive tumors.[220–222] Loss of E-cadherin and acquisition of VIM expression are events that characterize the epithelial-to-mesenchymal transition that occurs during embryogenesis.[223]

To begin to address this hypothesis, the invasiveness of epithelial-like (VIM–) and mesenchymal-like (VIM+) human breast cancer cells has been compared in the Boyden chamber and Matrigel outgrowth assays. Irrespective of hormone responsiveness, VIM+ cells exhibited significantly higher levels of both *in vitro* invasiveness and metastatic potential than did the VIM– negative group.[1,208] VIM expression was not detected in cells containing ER and was present in only some of the cell lines lacking ER, whereas E-cadherin was expressed functionally in all cell lines expressing ER as well as some that had lost ER expression and did not express VIM. These data suggest that the loss of E-cadherin expression is not linked to hormone independence but occurs earlier than VIM expression in the progression cascade. VIM expression also appears to be downstream of hormone independence.

E-cadherin expression, indicative of an epithelial phenotype, is associated with a compacted spherical morphology in VIM– cell lines when cultured in Matrigel.[224] E-cadherin is not present in any VIM+ cell lines. Absence of both E-cadherin and VIM is associated with a noninvasive cluster-type morphology. The NCI/ADR-RES cell line, derived from the MCF-7 cells by stepwise selection for increasing resistance to the drug adriamycin (see the previous section, Cells Selected for Resistance against Adriamycin), is interesting in this regard. Thought to be derived from the E-cadherin+/ER+/VIM– MCF-7 phenotype, this subline has lost ER and E-cadherin, gained VIM expression, and become significantly more invasive. Examination of additional adriamycin-resistant and vinblastine sulfate–resistant variants of the MCF-7 and ZR-75-1B cell lines shows that most, but not all, drug-resistant sublines expressed VIM.[225] Understanding of the relationships among drug resistance, VIM expression, and invasiveness may provide important clues for the optimization of chemotherapy for breast tumors.

The MCF-7/MIII and MCF-7/LCC1 variants retain ER and uvomorulin (UVO) expression, generally lack VIM expression, and exhibit somewhat lower levels of invasiveness than the VIM+ human breast cancer cells.[1,208] These observations support the hypothesis that these MCF-7 variants represent an intermediate point in the metastatic progression of breast cancer. The metastatic potential of the MCF-7/MIII and MCF-7/LCC1 cells, compared with the ER–/VIM– cell lines (e.g., MDA-MB-468),[61] however, suggests that metastatic potential may also develop independently of an event similar to the epithelial-to-mesenchymal transition.[226]

Oncogene Expression and *in Vitro* Metastatic Potential

The effects of oncogenes on mammary cell invasiveness have been examined. The *ras* oncogene is perhaps best studied and can induce the invasive phenotype in a variety of both human and rodent epithelial systems. Transfection of human bronchial epithelial cells transfected with v-Ha-*ras* increased both invasiveness *in vitro* and metastatic potential in the nude mouse.[227,228] In NIH/3T3 cells, transfection with either v-Ha-*ras* or genomic DNA containing various forms of activated *ras* also resulted in increased invasiveness across the amniotic membrane *in vitro* and metastatic dissemination *in vivo*.[229]

MCF-7 cells transfected with v-Ha-*ras* show increased *in vitro* invasiveness of Matrigel, increased migration potential, and increased recognition of laminin,[230] but no apparent increases in metastatic potential in nude mice.[231,232] Although both of the 184 sublines (A1N4 and B5) are transformed to a tumorigenic phenotype by expression of *ras* alone, only the initially more invasive A1N4 cells respond to *ras* transformation with increased invasiveness. The refractory nature of the 184B5 cells to *ras*-induced effects on invasiveness, despite the induction of tumorigenicity, suggests a possible lineage specificity for this response and begins to dissociate *ras* effects on tumorigenicity from invasiveness.

Differential induction of metastatic potential by v-Ha-*ras* has been previously reported.[182,231–233] Detailed analysis of a highly stable rat mammary subclone after *ras* transduction implicated rapid phenotypic diversification rather than direct effects on a cascade of metastasis-effector genes.[234] No changes similar to the epithelial-to-mesenchymal transition were seen after *ras* transformation of the A1N4 or B5 cells[213] or in the *ras*-transfected MCF-7 cells.[211] In contrast, combined transformation of 184A1N4–immortalized human mammary epithelial cells with v-Ha-*ras* and either *SV40T* or v-*mos* induces a VIM+, invasive phenotype indicative of the epithelial-to-mesenchymal transition event.[213]

The chemotactic activity and invasive property of the MCF-10A cells cotransfected with both Ha-*ras* and *erb*-b2 (MCF-10 HE cells) has also been further investigated using the Matrigel-based assays. MCF-10A HE cells showed tenfold higher invasiveness than the nontransfected cells, formed branching colonies in Matrigel, and showed a high cloning efficiency in soft agar (Thompson et al., *unpublished data*, 1999). These attributes are indicative of a VIM+ phenotype resulting from an event similar to the epithelial-to-mesenchymal transition.

CONCLUDING COMMENTS ON *IN VITRO* MODELS

Human breast cancer cell lines growing *in vitro* and as human xenografts *in vivo* have a central role in most basic and preclinical breast cancer research. They have been widely used to investigate the cellular and molecular events associated with endocrine responsiveness, malignant progression, invasiveness, and metastatic potential. With the increasing

restrictions being imposed on the use of vertebrate animals, and the relatively limited number of species that develop spontaneous mammary carcinomas, the emphasis on the *in vitro* use of human breast cancer cell lines seems likely to increase in the coming decades. Consequently, the introduction of additional representative human breast cancer cell lines, particularly hormone-responsive lines, and the realistic assessment and acknowledgment of the caveats associated with the use of *in vitro* models are critical.

Some Caveats Regarding the Use of *in Vitro* Models

Despite their widespread use and the considerable data arising from it, *in vitro* models have several potential limitations. Relatively few well-characterized ER+ cell lines exist. Although these cell lines tend to exhibit comparable estrogenic responses in the end points most widely applied, the extent to which these observations may be applied to all ER+ human breast tumors is unclear. Certainly, many of the most important attributes, such as growth inhibition by antiestrogens, are likely to closely reflect the human disease. In cases in which responses differ markedly from predicted or observed responses in humans, such as growth inhibition by physiologic levels of E2, a greater degree of caution is clearly warranted. A clear deficit in the range of breast cancer cell lines currently available is the relatively small number of ER+ and E2-responsive cell lines.

The majority of steroid-responsive cell lines have been established, not from solid tumors, but from malignant effusions. Although such effusions can occur with a 26% to 49% frequency in breast cancer patients,[235,236] they may not be fully representative of all solid tumors. Despite the likely metastatic origin of these cells, the ER+ cell lines from these sites are rarely metastatic *in vivo*, even in severely immunocompromised animals (see Chapter 22).

The most widely used cell lines have now been in use since the 1970s. Subtle changes may have been acquired during this period, and these may not adequately reflect changes that occur in human tumors in patients. Because the cells are clearly adapted to grow *in vitro*, the perturbations that have conferred this ability also may not occur in patients' tumors. Human breast tumors are highly heterogeneous and contain many subpopulations of cells with different phenotypic characteristics, including both ER+ and ER– cells.[237] In contrast, breast cancer cell lines are relatively homogeneous. This can be viewed either as an advantage or as a disadvantage. Although responses in a cell line may not fully reflect the response of a complex human tumor, they do provide the ability to study, in considerable detail and complexity, the responses of cells representative of tumor subpopulations.

In principle, cell lines are like any other experimental model. When their limitations are openly acknowledged and appropriately considered in experimental design and data analysis, they can provide useful and important tools. Otherwise, the risk exists of overinterpretation of data or the pursuit of a potential *in vitro* artifact. As a generalization, those observations from *in vitro* models that clearly reflect the human disease are more likely to reflect real events and lead to new insights into mechanistic processes. When these models are used to generate hypotheses for future testing in humans, the validity of the observation awaits completion of the human trials. Thus, major strengths of *in vitro* models include the ability (a) to study a specific cell type and elucidate the mechanism of its response to agents at the cellular and molecular level, (b) to identify mechanistic processes by comparing related cells with different phenotypic characteristics, (c) to facilitate further hypothesis generation and testing *in vivo* when cells are grown as xenografts, and (d) to generate hypotheses for testing in the ultimate model, the human being.

Establishment and Characterization of New Breast Cancer Cell Lines and Variants

Despite the presence of a number of breast cancer cell lines[2] and the emergence of new cell lines,[238] only three of those that are in common or widespread usage (the parental MCF-7, T47D, and ZR-75-1) are clearly estrogen dependent or estrogen responsive. Few of the established cell lines are metastatic in nude mice, and those that metastasize with a high frequency are generally ER–. Thus, new breast cell lines from malignant, solid metastatic (e.g., bone, lung), and normal tissues—specifically, steroid-hormone-responsive cell lines—are needed. Unfortunately, breast cancer cells from patients' tumors are notoriously difficult to establish *in vitro*.[239–241] The take rate for xenografts of tumor tissue in immunocompromised rodents also is relatively poor, although it is generally higher than that for direct *in vitro* growth.[242] Also, a well-characterized panel of nonmalignant breast epithelial cell lines that could be used for comparative studies is notably absent. Of those "normal" cell lines available, none is steroid hormone responsive.[172,173]

New breast cancer cell lines require careful description and characterization. When possible, the characteristics and history of the patient (e.g., age, sex, race, treatment) and the known characteristics of the tumor (e.g., histopathologic diagnosis, tumor grade, nodal status, ER and PR expression, S-phase/proliferativeness, and any other pertinent information) should be provided. The human origin of the tissues should be confirmed, and a karyotype and isoenzyme profile should be reported. Typical polymorphic enzymes analyzed include lactate dehydrogenase, glucose-6-phosphate dehydrogenase (EC 1.1.1.49), phosphoglucomutase-1 (EC 2.7.5.1), phosphoglucomutase-3 (EC 2.7.5.1), esterase D (EC 3.1.1.1), mitochondrial malic enzyme (EC 1.1.1.40), adenylate kinase (EC 2.7.4.3), and glyoxalase (EC 4.4.1.5).[14] These data are particularly useful in confirming the origin of a variant as being derived from its parent and in excluding contamination of a new cell line with cells of an established cell line. To this end, we have routinely used the services currently provided by Dr. Joseph Kaplan through the Cell Culture Laboratory of the Children's Hospital of Michigan in Detroit. This facility is currently maintained under National Cancer Institute contract to provide these services.

The general characteristics of the new cell line or variant should be clearly provided. This includes *in vitro* growth characteristics (e.g., culture conditions, cell doubling time, split ratio, and the passage number at which these data were obtained) and the hormone-receptor profile and endocrine responsiveness. A description of the morphology of the cells (e.g., ultrastructural analyses), particularly one that addresses tissue origin or evidence of differentiated function (e.g., secretory activity, production of milk proteins), is strongly encouraged.[2]

The tumorigenicity of a new cell line should be determined in at least one immunocompromised rodent model (preferably several) and reported. Different immunocompromised mice strains can exhibit different abilities to support xenografts, and the model in which tumorigenicity is assessed should be clearly indicated. The inability to form tumors in athymic nude mice may not indicate that the cells are nontumorigenic in other strains (e.g., *scid*; see Chapter 22). The histology of any arising tumors should be compared with that of the original tumor when possible. The presence of any metastases and their histology also should be documented. When available, any other pertinent data (e.g., oncogene expression or amplification) should also be provided.

The designation of a cell line or variant should follow the guidelines of the Tissue Culture Association and should reflect both the tissue of origin and the laboratory in which it was established.[243] For our variant cells, we have chosen to use a designation that appends our institution to that of the original (parental) cell line (e.g., MCF-7/LCC1).

Cell Culture Conditions

The choice of culture conditions often can inadvertently influence the experimental outcome. For example, for many years, insulin was routinely added to the cell culture media used to maintain breast cancer cells. Although insulin is a potent mitogen for many of these cells, it does not appear to be required for growth *in vitro* in serum-supplemented media for most human breast cancer cell lines. Insulin can down-regulate ER expression, however.[244] Insulin and EGF have been added to serum-free media for breast cancer cells,[245] and both can influence the growth-inhibitory effects of antiestrogens.[246,247] Phenol red is widely used as a pH indicator in cell culture media. A contaminant in phenol red is known to be estrogenic, and this activity can alter both the growth and antiestrogen responsiveness.[248,249]

Serum contains various growth factors and steroid hormones. The steroids can be readily removed by treatment with charcoal-coated dextran.[250] MCF-7 cells also can use the steroid sulfates present in serum.[251] These steroid sulfates can be removed by prior treatment with sulfatase.[250] The growth factors and other proteins can be chemically inactivated to produce a growth factor–free serum.[252] The concentration of serum used also can be important. The dose-response relationship for antiestrogens is altered significantly by serum concentration.[253] We have found a final concentration of either 5% fetal calf serum or 5% charcoal/dextran-stripped serum to provide appropriate *in vitro* growth characteristics for most human breast cancer cell lines. The concentrations of steroids, growth factors, and other constituents in serum may vary considerably from batch to batch.

Estrogens remain within cells for several days,[254] and when stripping cells of endogenous estrogens, one must often thoroughly wash cell monolayers and maintain cells for several days in the absence of estrogens. We routinely wash cells at least three times with phenol red–free media supplemented with 5% dextran/charcoal-stripped serum and maintain the washed monolayers in this medium for a further 3 to 5 days to ensure adequate removal of endogenous steroids.[14,62]

The cell-seeding density also can have considerable effects on cellular growth and metabolism. Cells seeded at different densities have previously been demonstrated to exhibit both different cell population doubling times and differences in methotrexate poly-γ-glutamate formation.[99] In many instances, one must closely control for seeding density, proliferative capacity, confluence, and serum batch. The handling of cells, including duration of trypsinization and time at room temperature during passage or treatment, may also be important. For some cell lines, a trypsinized cell suspension must be passed through a sterile needle to generate a single cell suspension.

These examples indicate the general importance of closely controlling cell culture conditions. The reader is referred to several excellent books on tissue culture techniques for a more detailed description of cell culture procedures.[255–257]

SUMMARY

The development of stable cell lines derived from malignant and normal human breast tissue has been of considerable use in breast cancer research, and such cell lines continue to occupy a central position in basic breast cancer research. These cell lines provide the ability to conduct studies that could not easily be performed in experimental animals or human beings. The ease of use, relatively low cost of maintenance, general reproducibility of phenotype, and ability to mimic properties seen in tumors in patients are considerable advantages. However, the use and applicability of cell lines are not without limitations. For example, cell lines cannot be used to reliably predict *in vivo* toxicity or to assess the toxicologic properties of new agents. Cell lines also may be ineffective in establishing mechanisms of drug metabolism or in elucidating critical tumor-host interactions. Their metabolic adaptations to *in vitro* growth may not reflect adaptations that occur *in vivo*. Nevertheless, breast cancer cell lines have been used successfully for many years to generate new hypotheses, screen new agents, and study the biology of breast cancer. Many cell lines have the advantage of being tumorigenic and thus can facilitate further studies *in vivo* in experimental animals. Provided their limitations are freely acknowledged, human breast cancer cell lines will continue to provide one of the most powerful tools in breast cancer research.

ACKNOWLEDGMENTS

This work was supported in part by grants NIH R01-CA/AG58022, NIH P30-CA51008, and NIH P50-CA58185 (Public Health Service) and USAMRMC (Department of Defense) BC980629 and BC980586.

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Breast Cancer Research and Treatment **46**: 255-278, 1997.

Issues in experimental design and endpoint analysis in the study of experimental cytotoxic agents *in vivo* in breast cancer and other models

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Key words: xenografts, breast cancer, cell lines, resistance, cytotoxic drugs, synergy

Summary

Considerable effort has been placed into the identification of new antineoplastic agents to treat breast cancer and other malignant diseases. The basic approaches, in terms of model selection, endpoints, and data analysis, have changed in the previous few decades. This article deals with many of the issues associated with designing *in vivo* studies to investigate the activity of experimental and established compounds and their potential interactions. Endpoints for both *in situ* and excision assays are described, including approaches for determining cell kill, tumor growth delay, survival, and other estimates of activity. Suggestions for approaches that may limit the number of animals also are included, as are possible alternatives for death as an experimental endpoint. Other concerns, such routes for drug administration, drug dosage, and preliminary assessments of toxicity also are addressed. Statistical considerations are only briefly discussed, since these are addressed in detail in the accompanying article by Hanfelt (Hanfelt JJ, Breast Cancer Res Treat 46:279-302, 1997). The approaches suggested within this article are presented to draw attention to many of the key issues in experimental design and are not intended to exclude other approaches.

Introduction

The disseminated nature of breast cancer and the development of crossresistant tumors are the primary causes of failure of current therapies. By the time many tumors are detected, there is a high probability that metastatic lesions will be present, many of which may already contain resistant subpopulations [1]. Not surprisingly, there is substantial interest in the identification of novel cytotoxic and endocrine agents for the treatment of breast cancer.

A systemic approach is required to eradicate the majority of metastatic breast disease, and this remains primarily in the form of cytotoxic chemotherapy or endocrine manipulation. The latter began with the initial studies on oophorectomy by Beatson [2] and was followed by the administration of high dose estrogens. These were largely replaced by the development of antiestrogens,

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Table 1. Several of the human breast tumor cell lines used as xenografts in the current NCI drug screening program*

Cell Line	ER status	Citation
BT-549	negative	**
Hs578T	negative	(106)
MCF-7	positive	(107)
MCF7/ADR (multidrug resistant)	negative	(39,40,106)
MDA-MB-231 (metastatic)	negative	(108,109)
MDA-MB-435 (metastatic)	negative	(108-110)
T47D	positive	(111)

* Kindly provided by Dr. Joseph Mayo.

** There is no primary citation for this cell line listed by the provider (ATCC, Rockville, MD), who indicate in their "Catalogue of Cell Lines and Hybridomas" that the cells were derived from a papillary invasive ductal tumor in a 72-year old patient. Metastatic disease was found in three of seven regional lymph nodes. The originators were Drs. W.G. Coutinho and E.Y. Lasfargues. A discussion of other human breast cancer cell lines can be found elsewhere [112].

initially the triphenylethylene Tamoxifen [3] and, more recently, introduction of the steroidal anti-estrogen ICI 182,780 [4]. Other endocrine strategies include the use of inhibitors of estrogen biosynthesis, primarily aromatase inhibitors, and the use of LHRH agonists and antagonists.

The use of chemotherapeutic agents in the management of neoplastic disease began with Rhoad's description of the use of nitrogen mustard for the treatment of Hodgkin's lymphoma [5]. The number of cytotoxic agents available has increased substantially over the intervening years, with breast tumors generally exhibiting good overall response rates to several cytotoxic drug combinations. While chemotherapy can produce gains in overall survival, most patients with metastatic breast cancer will eventually recur. Many reasons may account for this failure, including poor dose scheduling, inappropriate combinations of drugs and the emergence of cell populations resistant to the antineoplastic agents.

Animal models provide one approach for the optimization of drug scheduling and for the identification of novel compounds that exhibit promise in such *in vitro* prescreens as those currently utilized by the National Cancer Institute [6]. For many years a primary *in vivo* screen, utilizing the L1210 and P388 murine leukemias, was an integral part of the NCI's preclinical drug development program. However, the lack of a significant representation of solid human tumors drew criticism, and partly explained the weakness of the screen to identify new agents active against the more common solid tumors, including breast cancer. Perhaps not surprisingly, the screen appears to have been more successful in identifying agents active against hematologic malignancies. Some drugs with well established clinical efficacy (busulfan, hexamethylmelamine) fail to demonstrate substantial activity in the L1210/P388 screen [7,8].

The P388 and L1210 *in vivo* screen has largely been replaced by panels of disease specific human tumor cell lines (prescreen) and xenografts (primary screen). While there are relatively few ideal *in vivo* models for breast cancer, several of the available human cell lines fulfil some of the requirements for screening, *e.g.*, stable phenotype, high tumor take rate, predictable and reproducible kinetic properties. Several of those currently used by NCI are shown in Table 1.

Some considerations for choice of model, scheduling, dosage, and endpoint will be discussed below. The discussions and issues raised are to draw attention to different approaches to experimental design. As such, these should be considered in the light of the other articles on experimental design and data analysis in this issue and elsewhere. Some issues are generic, while others are more directly applicable to screening cytotoxic activities, and may be of lesser relevance to testing chemopreventive and endocrine agents. It is hoped that the topics discussed will assist investigators to consider key issues in experimental design. However, these are provided only as suggestions. There are many ways to identify potentially active compounds and drug combinations, and these are constantly being

modified by improvements in both cellular models and approaches to the use of animals in biomedical research.

Log cell kill and tumor kinetics in cytotoxic cancer chemotherapy

Early attempts to modify cytotoxic therapies were largely empirical. The initial criteria for designing cytotoxic therapies were based on the observations of Skipper, who demonstrated that cytotoxic drug-induced cell kill follows a similar kinetic pattern to that established by Arrhenius at the turn of this century for the killing of bacteria. The log cell kill hypothesis states that cytotoxic drugs kill cells by first order kinetics [9]. Thus, a constant proportion of the cells will be killed regardless of the size of the cell population. For many cytotoxic drugs a 4-log cell kill is achievable and would eradicate a tumor population of 10^3 cells and have a one in ten chance of eliminating a population of 10^4 cells [10]. However, clinically detectable primary tumors have a cell population frequently in excess of 10^9 cells. Many metastases also could contain cell populations greater than 10^4 cells. While theoretically sound, log cell kill can be affected by several biological parameters, including the presence of *de novo* resistant cells, the degree of tumor vascularity, and other factors that may affect drug perfusion and metabolism.

The principles elucidated by Skipper were further modified by Norton & Simon, who applied Gompertzian growth kinetics to tumor cell populations [11]. This clarified the inverse relationship between growth fraction and tumor size. The ability of a cytotoxic drug to inhibit tumor cell growth was determined to be directly related to the tumor's growth rate, which also is related to tumor volume [12]. The tumor mass killed by a cytotoxic treatment is proportional to the growth fraction multiplied by the total tumor volume [12].

A Gompertzian kinetic growth pattern produces a growth fraction that ultimately decreases exponentially with time. This inverse relationship between tumor size and growth fraction implies that micrometastases should be more kinetically sensitive to cytotoxic chemotherapy than larger tumor masses [13,14]. Thus, early intervention when the tumor mass is small should provide the greatest opportunity for induction of remission. Metastases tend to exhibit a more rapid tumor doubling time (T_D) than primary tumors, particularly for the common solid tumors like those of the breast [15].

There are a number of factors that contribute to the apparent T_D of any tumor. These include the rate of cell production, the size of the growth fraction, cell recruitment from G_0, and the rate of cell loss. Cell loss includes shedding of cells into other compartments, *e.g.*, metastasis, differentiation to a non-proliferating cell type, or entry into prolonged G_0 and apoptotic cell death. The high proportion of non-proliferating or normal cells present in many solid tumors, and the frequently high rate of cell loss, generally produce tumors with a long T_D. The rate of cell loss may be as high as 80% of the rate of cell production [16].

Growth kinetics in human tumors and animal models

The kinetic parameters of tumor growth represent one of the major differences between animal models and the human disease. While Gompertzian kinetics apply to experimental tumors and those in patients, the T_Ds and growth fractions are frequently quite different. For example, many human breast tumors exhibit long T_Ds and often small relative growth fractions. In marked contrast, human breast tumor xenografts generally have short T_Ds and high growth fractions. Estrogen-treated MCF-7 tumors (ER-positive) have T_Ds of approximately 10-12 days [17-19] compared with over 100 days for many tumors in patients [20]. We have generated several estrogen-independent MCF-7 variants. These have T_Ds as long as 100 days when grown in the absence of

estrogen, but grow as rapidly as parental MCF-7 tumors in estrogen-supplemented mice [17-19]. MDA435/LCC6 cells (ER-negative, ascites variant of MDA-MB-435) growing as solid tumors have much shorter T_Ds of 3-5 days [21]. Most of the breast cancer xenografts used in the current NCI screen have mean T_Ds of 2-10 days.

The differences in kinetic properties between xenografts and tumors in patients would tend to make the xenografts more sensitive to agents with a strong cell cycle/cell phase specificity. For the purposes of a primary *in vivo* screen for novel agents, a limited overestimate of activity may not be a major concern. For studies to optimize scheduling or combinations of established drugs, the relative sensitivity of the *in vivo* screen is a concern only if the model is either too sensitive or too resistant to a combination, when it will become difficult to assess interactions. Most of these concerns are readily addressed by a careful choice of *in vivo* model(s). Various schedules have already been identified for the established drugs; examples are provided in Table 2.

The dose response relationship and dose intensity

The relationship between treatment and response is described by:

$$k = C \times t$$

where C = concentration; t = time. Thus, response should be approximately equivalent where C x t values (area under the concentration time curve) are equivalent. This can enable the design of clinically relevant *in vitro* analyses of established drug combinations based on pharmacokinetic measurements previously obtained in patients or animals. Clinical studies can utilize the reasonable across-species dosage relationship of mg/m^2 to estimate dose from data obtained in preclinical animal screening. The doses may require some further modification, since the serum half-life of some drugs can be longer in man than in rodents [22]. One approach is to use 1/10th the maximum tolerated dose (MTD) in rodents as the approximate starting dose for a Phase I trial in humans [23]. Where possible, it may be better to use a dose that produces comparable pharmacokinetics, since this can increase the predictability of the mouse xenograft-to-human tumor model [24].

The pharmacokinetics for cytotoxic drugs are frequently similar in mice and men [25]. For many drugs, the mouse LD_{10} also approximates the MTD in humans when expressed as mg/m^2 [26]. However, there are exceptions. The C x t values at the LD_{10} are higher for mitomycin C, vincristine, and cyclophosphamide, and lower for methotrexate and 5-fluorouracil, in mice when compared with humans (reviewed in [26]).

In clinical practice, a narrow therapeutic index is frequently responsible for the reduction of dosage due to side effects. However, the steep dose response curve for most cytotoxic drugs implies that even a small perturbation in dosage may produce a significant change in response. The effects of alterations in dosage on clinical response has been widely reviewed [22,27]. It has been suggested that a major contributing factor to the failure of many treatments is the *ad hoc* reduction of drug dosage [22].

It has been widely acknowledged that the most effective treatments involve a high dose intensity chemotherapeutic regimen. This is partly based on the steep dose response relationship for most cytotoxic drugs and various other clinical observations. Dodwell *et al.* [27] have reviewed the published data, and reexamined the role of dose intensity in response, for a number of the more common malignancies. While they conclude that high intensity regimens can produce significant advantages in disease free survival, clear demonstrations of increased overall survival are obtained much less frequently. The relationship between dose intensity and response often varies with both drug, tumor model, and disease. Animal models provide a safe and logical means to explore this and related issues, rather than attempting to identify appropriate or potentially dangerous schedules directly in patients.

Table 2. Examples of dosages and schedules for several established cytotoxic agents. The tumor models against which the drugs were tested were of various origins, and not exclusively breast. Some drugs that are not widely used in breast cancer are included, since these may be useful as controls for establishing the extent to which a tumor model reflects the human disease. Toxicity can vary with strain, sex, age and other parameters, and the information in this Table reflects that diversity.

Drug	Dose[1]	Route	Schedule[2]	Toxicity	Citation
Adriamycin	6 mg/kg	i.v.	4,6,8	ND[3]	[67,113]
	6 mg/kg	i.v.	1,5,9	ND	[114]
	6.8 mg/kg	i.p.	single dose	none[4]	[21]
	8.5 mg/kg	i.p.	single dose	LD[5]	[21]
	24 mg/m^2	i.v.	single dose	MTD	[25]
Ara-C[6]	40 mg/kg	s.c.	24 hr infusion	none	[115]
	50 mg/kg	i.p.	1-7	alopecia	[116]
BCNU	18 mg/kg	i.p.	single dose	LD	[21]
Methyl-CCNU	18 mg/kg	i.p.	single dose	MTD[7]	[71]
Cyclophosphamide	35 mg/kg	i.p.	single dose	alopecia	[116]
	60 mg/kg	i.p.	0-4, 7-11	ND	[117]
	100 mg/kg	i.v.	1,5,9	ND	[114]
	143 mg/kg	i.p.	single dose	none	[118]
	200 mg/kg	i.p.	1,15	LD	[119]
	286 mg/kg	i.p.	single dose	LD	[118]
	290 mg/kg	i.p.	single dose	none/LD	[81]
5-Fluorouracil	32 mg/kg	i.p.	5,6,7,8	ND	[72]
	40 mg/kg	i.p.	1-4, 15-18	LD	[119]
	50 mg/kg	i.p.	0-4	ND	[117]
	60 mg/kg	i.v.	1,5,9	ND	[114]
	180 mg/m^2	i.p.	single dose	MTD	[25]
Ifosfamide	150 mg/kg	i.v.	single dose	none	[77]
	300 mg/kg	i.v.	single dose	none	[77]
Melphalan	12 mg/kg	i.p.	single	ND	[120]
	12 mg/kg	i.p.	0,4	ND	[120]
Methotrexate	4 mg/kg	i.p.	1,4,8,11,15,18	LD	[119]
	15 mg/kg	i.v.	1-5	ND	[119]
	20 mg/kg	i.p.	0-3	ND	[117]
Mitomycin C	2 mg/kg	i.v.	1,15	LD	[119]
	2.5 mg/kg	i.v.	single dose	LD	[121]
	4.5 mg/kg	i.p.	single dose	none	[21]
	5 mg/kg	i.p.	single dose	ND	[122]
	18 mg/m^2	i.p.	single dose	MTD	[25]
Cis-Platinum	4 mg/kg	i.p.	1,5,10	ND	[123]
	4 mg/kg	i.v.	1,5,10	ND	[123]
	7.5 mg/kg	i.p.	single dose	none	[21]
	10 mg/kg	i.p.	single dose	ND	[123]
	27 mg/m^2	i.p.	single dose	MTD	[25]
Taxol	20 mg/kg	i.p.	single dose	none	[21]
Taxotere	15 mg/kg	i.v.	4,6,8	MTD	[67]
Vinblastine	3 mg/kg	i.v.	1,15	LD	[119]

1 = Dose/injection; 2 = Schedule is given as days unless otherwise indicated; 3 = Not defined or not described; 4 = None reported or no deaths; 5 = Lethal dose (one or more deaths attributed to drug-induced toxicity); 6 = 1-β-D-arabinofuranosylcytosine; 7 = Approximate MTD as defined by the investigators.

General considerations in experimental design

Choice of host for xenografts

There are several considerations relating to the choice of immunocompromised host, including the degree of immune-competence and the ability of the strain to support the growth of the tumor. For syngeneic tumors the choice of host should be obvious. However, the choice of host for xenografts is more complex. The host not only should facilitate the growth of the tumor but also should enable the tumor to maintain a biologically relevant phenotype. It also is useful if the selected model enables some estimation of the selectivity of drug action and the determination of potentially lethal toxicities. For most of the breast cancer cell lines/xenografts available, the *nu/nu* mouse is sufficient, irrespective of the background strain [28].

The potential for immunological modulation to contribute to tumor response to combination chemotherapy is an important consideration. This is particularly relevant when the combination includes biological response modifiers that can influence the activation of cells associated with cell mediated immunity, *e.g.,* interferons and interleukins. In some cases, it may be desirable to attempt to either eliminate or exclude effects on the immune competence of the host. Thus, the choice of an appropriate immune-deficient strain may become paramount. Since the different immunobiologies of available rodent hosts have been recently reviewed [28], they will not be further discussed.

Choice of appropriate tumor model

The model used for the screening of a drug suspected of activity against a particular tumor should reflect the biological properties of that tumor as closely as can reasonably be achieved. For example, a drug active against a leukemia with a short T_D, high growth fraction, and relatively short cell cycle time would be less likely to demonstrate activity in a screen against a breast tumor with a longer T_D, small growth fraction, and long cell cycle time. Thus, choice of an appropriate model to screen combinations of agents with known pharmacological and kinetic requirements should (where possible) closely reflect the major biological properties of the human disease. To some extent the model also should reflect the requirements of the drug. For example, there would be limited value assessing a drug expected to show no crossresistance to P-glycoprotein without including a model that expresses P-glycoprotein.

Selecting a breast tumor model for screening cytotoxic compounds can be problematic. Relative to the murine ascites models, most solid breast tumors exhibit a relatively slow T_D. For example, MCF-7 tumors have a T_D of approximately 10-12 days when growing in appropriately estrogen supplemented animals [19]. While this may be acceptable for screening antiestrogenic or chemopreventive agents, estrogenic supplementation can alter the activity of some cytotoxic drugs [29,30]. In general, the most rapidly proliferating human breast tumor xenografts do not express estrogen receptors. MDA435/LCC6 tumors have T_Ds of 2-3 days. Since the parental cell line (MDA-MB-435) was obtained from a patient who had not received chemotherapy, these models may be useful in screening cytotoxic agents for activity against breast cancer [21]. The MDA-MB-231 cell line also has a comparably rapid T_D *in vivo* (R. Clarke, unpublished observations). The more rapid T_Ds of ER-negative xenografts broadly reflect the characteristics of such tumors in patients, where ER-negative tumors tend to have shorter T_Ds [31].

From a purely practical viewpoint, relatively slowly proliferating breast tumors can produce significant problems in logistics and experimental design. The slower growing tumors frequently exhibit significant intertumor variability and can require substantial numbers of animals to enable meaningful statistical analysis of data. Determining the period at which a tumor is considered

"cured" also can become problematic. Even the more rapidly proliferating solid tumors with T_D = 48 hr may require up to four months of post treatment observation to establish "cure" [32].

It is unlikely that any one tumor model will adequately represent the major biological characteristics of a particular malignancy. Thus, the use of a series of tumors (where appropriate/available) may be required to determine the sensitivity of a particular neoplastic disease to a either a single or a combination chemotherapy regimen. However, this must be considered in the context of reducing animal usage, cost, and the value of the additional data obtained.

For breast cancer, there are several potential models available for screening (Table 1). Most of the ER-positive models require estrogenic supplementation for tumorigenicity or maximal growth. We have generated ER-positive models that will grow without supplementation, but the respective T_Ds are relatively long [19]. While this may be more representative of breast tumors in general, this characteristic is inappropriate for screening cell cycle or cell phase specific cytotoxic compounds. We also have developed an ascites model based on a variant of the MDA-MB-435 cell line (MDA435/LCC6). The pattern of response to a variety of cytotoxic drugs appears to reflect closely that seen in breast cancer patients [21]. For example, breast cancers in general respond poorly to nitrosoureas [33], as do MDA435/LCC6 ascites to BCNU. Etoposide also does not produce long term survivors, and this drug generally has been ineffective as a single agent in breast cancer [34,35]. Adriamycin [34], mitomycin C [36], and taxol [37] are among the most effective single agents in previously untreated breast cancer, and all of these drugs produced long term survivors in mice bearing the MDA435/LCC6 ascites. The characteristics of several breast cancer xenografts have been reviewed elsewhere [28].

Another example of the choice of tumor model is in studies to evaluate P-glycoprotein reversing agents, compounds which may have significant potential in some breast cancer patients [38]. For these types of analyses the choice of tumor model is critical. Cells to be used as xenografts should be transfectants rather than selected for resistance, since selection can produce multiple unrelated resistance mechanisms. For example, MCF-7^{ADR} (selected for resistance to adriamycin [39]), but not MDR1-transduced MCF-7 cells (CL 10.3), are cross resistant to Tumor Necrosis Factor [40]. Since both adriamycin and Tumor Necrosis Factor can inhibit cells by the generation of free radicals [41,42], this cross resistance in $MCF7^{ADR}$ cells suggests the presence of functional adriamycin resistance mechanisms in addition to P-glycoprotein, including altered expression of manganous superoxide dismutase [40]. These cells also exhibit increased glutathione transferase and topoisomerase II activities [43,44]. The use of transfected cells allows for a clearer interpretation of the data.

Cells concurrently expressing multiple resistance mechanisms may more closely reflect the drug resistance that occurs in patients [38], but interpreting responses in a mechanistic light may be difficult. This does not invalidate their use where the purpose is simply to screen compounds for potential antineoplastic activity. Indeed, the choice of a series of models that are too sensitive will likely identify compounds with limited activity in patients, whereas active compounds identified in otherwise resistant models may have a higher probability of being active in patients [45]. This is likely to be true in principle, but the extent to which it applies will depend upon whether the resistance mechanisms operating in the tumor model contribute significantly to the resistance phenotype in patients.

It is apparent that there are two potential types of screening approaches, each with different objectives that will result in different choices of models. Where a broad based, non-mechanism oriented screen is required, a disease-specific panel of xenografts with widely differing but biologically relevant phenotypes is likely to be optimal. Knowledge of the pattern of response to a series of established drugs, for each component of the panel, is required. Such a panel might be

expected to contain both sensitive and resistant models (see Table 1 for examples included in one possible panel for breast cancer). For a mechanism or structure/function based screen, the choice of components is likely to depend upon assumptions inherent in the mechanism. For example, where a specific target is identified, the panel may contain several models with different levels of expression of the target, *e.g.,* P-glycoprotein, multidrug resistance related protein [21,38,46,47]. In many cases, this requirement may be most effectively met by a series of transfected cell lines and their respective control populations.

Phenotypic stability

The stability of the phenotype is a critical determinant for tumor model selection. Some tumor xenografts may require periodic cycles of *in vivo/ in vitro* growth in order to maintain the ease of reestablishment *in vitro* for some excision cytotoxicity assays. Prolonged *in vivo* growth of some established cell lines can result in significant phenotypic alterations. We have described the isolation of hormone-independent sublines of the estrogen-dependent MCF-7 human breast cancer cell line following prolonged selection *in vivo* [17,18]. While responses to antiestrogens remain unaltered [18,48], there are significant changes in their responsiveness to estrogens [17,18,48]. For many cell lines, this problem can be overcome by using cells within a limited number of passages ($\leq$10) from a single frozen stock of cells. The frozen stock should be from a single passage of cells with a well characterized phenotype.

The intertumor stability of the growth patterns and cell cycle profiles also may be important considerations. A high degree of variability in intertumor growth fractions could significantly influence the reliability or ease of data interpretation. The stability of the metastatic potential must be well defined. Cells with an unpredictable metastatic capacity may alter tumor burden and affect survival and/or the host's sensitivity to the toxicity of cytotoxic treatments.

Therapeutic index and dose scheduling

The difference in the dose response curves of normal and neoplastic tissues, often referred to as the therapeutic window or therapeutic index, is widely applied in the clinical pharmacology of cytotoxic drugs [22,49-51]. This difference in drug sensitivity enables the administration of sufficient drug to produce cytotoxic effects in the tumor, but not to induce significant and irreversible toxicity to normal cells. Unfortunately, many cytotoxic drugs exhibit a steep dose response curve with a small therapeutic index. In many cases, the development of unacceptable toxicity is the dose limiting factor for cytotoxic chemotherapy.

The sensitivity of normal cell populations reflects the rapid growth rate and high growth fraction of some cells. For example, cells in normal bone marrow and the intestinal crypts have respective thymidine labeling indices of 30%-70% and 12%-18% [15]. These values are generally higher than observed in most solid tumor cell populations. This accounts for the high incidence of hematopoietic and gastrointestinal side effects associated with many chemotherapeutic regimens. Granulocytes are highly susceptible to cytotoxic agents because of their high growth fraction and short lifespan, but a pool of non-cycling stem cells ensure repopulation [15]. Generally, the purpose of dose scheduling is to administer the second and subsequent treatments at times that will allow some normal stem cells to evade the drug and enable repopulation to occur without permanent damage to bone marrow. Examples of some drug schedules for rodents are provided in Table 2.

Drug administration in vivo

The route of administration can influence drug pharmacokinetics, toxicity, and antitumor activity. For example, injection *i.v.* introduces the drug directly into the blood, with a relatively rapid exposure of hematopoietic stem cells. Adminis-

tration *s.c.* or *i.p.* would be expected to produce a slower drug equilibration with this compartment, and produce a delayed or reduced myelosuppression. However, local tissue damage could be increased relative to the *i.v.* route. The potential toxic/pharmacokinetic differences associated with routes of administration can be controlled by careful planning. For example, while an *i.v.* bolus of an agent may produce unacceptable toxicity, the same dose in mg/kg body weight can occasionally be administered either by infusion, *s.c,. p.o.,* or multiple lower doses given *i.v.,* with significant alterations in host toxicity.

For the majority of solid tumors, an *i.v.* administration most closely reflects the clinical administration of the drug. However, for experimental drugs, the route of administration will vary primarily with the physico-chemical properties of the drug. The *i.v.* route is generally limited to water soluble compounds with a pH >4.0 and <8.5. Water-insoluble drugs can be administered either *s.c.,* provided they do not produce unacceptable local damage, or *p.o.* if they have sufficient chemical stability. For steroid hormones and antihormones that can require sustained delivery, *i.p.* or *s.c.* depots in peanut oil, *s.c.* Alzet mini pumps (Alza Scientific, Palo Alto CA), cholesterol-based slow release pellets (Innovative Research of America, Sarasota, FL) or silastic pellets all can produce appropriate plasma levels of drug for sustained periods of time.

The timing of administration also is an important consideration. Initiation of treatment within a few days of tumor cell inoculation may produce evidence of activity, when administration to established tumors indicates inactivity. For cytotoxic agents, administration within a few days of cell inoculation is often inappropriate. Treatment of established tumors, where these are clearly palpable and fall within a predefined size range, is generally more appropriate and allows for assessment of the most widely used endpoints. This size should not be so large as to influence response to the drug. Early administration of drug is usually appropriate when the tumor is directly xenografted from another animal and has a rapid T_D of only a few days, or perhaps when sensitivity is required in a primary screen. Another exception is for the testing of chemopreventive agents, *e.g.* antiestrogens or retinoids, which could be given to high risk women without evidence of clinically detectable disease. With these compounds, chemopreventive/chemosuppressive activity against low tumor burdens, *e.g.* recently inoculated tumor cell suspensions, may more closely reflect the proposed clinical use. Where initiation of treatment around the time of tumor cell inoculation is justified, *i.e.* before the appearance of palpable tumors, the more common endpoints include time to tumor appearance and tumor incidence.

Pharmacokinetics and toxicity in drug combination studies

There are a number of pharmacokinetic considerations that can influence activity of a drug *in vivo* when drugs are combined. The biological activity of one agent may alter the metabolism, absorption, distribution, or toxicity of another. Insulin can alter the metabolism and subsequent cytotoxicity of methotrexate [52]. Prednisolone reduces host toxicity and enhances the antitumor effects of nitrogen mustard, melphalan, and chlorambucil [53]. In some experimental tumor systems progesterone can reduce the systemic toxicity of chlorambucil [53]. Martin *et al.* [54] have reported that adriamycin, lomustine, carmustine, semustine, and vincristine can increase melphalan uptake in L1210 cells. In patients with advanced breast cancer, the toxicity of a combination of cyclophosphamide, methotrexate, and 5-fluorouracil is reduced by fluoxymesterone [48]. Bleomycin cytotoxicity is increased by several membrane-acting drugs [55]. Illiger and Herdrich [56] have extensively reviewed many of the drug interactions encountered in cytotoxic chemotherapy.

The purpose of some experimental designs is to specifically test the interactions of drugs. For

example, there is considerable interest in the generation of drugs that may reverse the efflux activities of P-glycoprotein, the glycoprotein product of the MDR1 gene [57]. There is clear evidence that some reversing agents, if not all, alter the pharmacokinetics of cytotoxic drugs [58]. This may reflect modulation of normal P-glycoprotein function in the liver and other tissues, leading to an effective increase in drug exposure. Thus, increased activity of a cytotoxic agent requires careful evaluation.

One simple way to control for such effects is to compare equitoxic doses, *e.g.* the cytotoxic drug alone and in the presence of the reversing agent using schedules each of which produce an MTD. While this is a reasonable approach for pilot studies, directly assessing the pharmacokinetics of the cytotoxic drug with and without the reversing agent is definitive. Ultimately, such pharmacokinetic data may be required to clearly demonstrate that effects are not simply due to perturbations in the cytotoxic drug's mean serum concentration/plasma residence time.

Estimation of drug dosage and choice of starting dose/dose range

Where established drugs are to be used, well documented protocols are available from the literature (Table 2). This is, at best, a general guide, since some rodent strains may be more or less sensitive to the toxic side effects of specific agents.

Identifying a dose regimen for unknown or experimental agents is frequently empirical. Often the initial studies are performed to obtain estimates of the MTD/LD_{10}. A simple dose escalation study with a limited number of animals per group is a common strategy. The choice of starting dose to obtain an MTD estimate could be based on one of several criteria, such as the known *in vitro* toxicity, or toxicity of a closely related compound. Where agents under investigation are natural products or analogues thereof, *e.g.* phytochemicals, the levels of exposure in human populations may be available and enable estimation of the starting dose. One simple approach, where resources are limited, is to do a pilot (dose range finding) study, using a broad range of doses but a small number of animals (one or two animals per dose). This has the advantage of limiting the number of animals that may be exposed to particularly toxic/lethal doses when the intent is to rapidly establish a toxic dose. A second, more definitive, follow-up study can then be done to obtain the MTD, using a limited number of mostly sublethal doses with larger numbers of animals per group. Animal usage can be further limited by restricting these follow-up studies only to those drugs that show antineoplastic activity and are identified for further evaluation in subsequent secondary screening.

For preliminary toxicological analyses, *e.g.*, estimates of MTD, all animals should be monitored twice daily at a minimum. Recording animal body weights and food consumption twice weekly can identify the onset of some toxicities that are not immediately apparent. Loss of 10% of the starting body weight, or failure to gain body weight at the same rate as controls, provide useful endpoints for estimating an MTD. Many cytotoxic agents will produce more immediate effects, which are often apparent from the altered behavior of the animals, *e.g.* crouching, somnolence, or reduced activity. These also can be used as endpoints to establish an MTD. Such effects may be transient, with the animals recovering within several hours if no further drug is administered, or they can persist and lead to morbidity and death if prolonged over several days or weeks.

Where drug related deaths occur within several hours of administration, these data should not be used to establish the MTD, since toxicity could merely reflect sensitivity to the peak plasma concentration resulting from the bolus. Rather, the dosage and/or schedule should be modified, *e.g.* using lower doses and perhaps more frequent administration. This pattern of toxicity, *i.e.* schedule-independent with peak plasma level toxicity, has been referred to as category III [59].

Other categories reflect whether toxicity is schedule dependent (category I) or independent (category II, where total dose determines toxicity) [60,61].

Hematologic toxicity is often assessed as a toxicological endpoint, but the transient suppression of some cell populations, and the timing of their likely recovery, need to be considered. It can be useful to measure white blood cell counts and hematocrits twice weekly, or as appropriate, on two or more individuals in each group. In many cases this can be done by retroorbital bleeding. However, the same animal should not be bled repetitively, since this can be sufficient to influence the parameters under investigation, independent of the treatment. It should also be noted that hematopoietic toxicity is not always evident from peripheral blood assays, and may require spleen colony formation analyses [49]. Additional evidence of toxicity may be apparent on determining other parameters, *e.g.* SPGT, total bilirubin, BUN, creatinine. Such detailed blood analyses are usually restricted to more intensive toxicological studies, where identifying the dose limiting or lethal toxicity is required. However, if the aim is to obtain an approximate MTD in a pilot study, it can be informative to sacrifice moribund or clearly affected animals by terminal bleeding under appropriate anesthesia. The blood can then be used for a wider panel of preliminary tests. It is usually routine to sacrifice all animals at the end of the study and perform necropsies with subsequent examination of the major tissues and organs for evidence of gross and/or microanatomic toxicity.

Site of inoculation, tumor-host interactions, and drug delivery

The site of tumor cell inoculation can significantly alter tumor growth and metastatic potential [62-65] and has previously been discussed in detail [28]. Cells re-inoculated into the site of the original tumor (orthotopic implantation) produce metastatic lesions more frequently than those inoculated elsewhere [64]. We routinely use the mammary fat pad area as the preferred inoculation site for mammary tumors [17]. Where orthotopic implantation is either inappropriate or not required, *s.c.* inoculation into the flank facilitates good responses for cytotoxic agents and *s.c.* inoculation into the back facilitates good responses for irradiation regimens [66].

The "tumor bed effect", which relates to modifications in the tissue at the site of implantation, has been most widely studied in relation to radiosensitivity but can also influence response to cytotoxic drugs [54]. Some tumor models utilize inoculation into preirradiated subcutaneous sites. Prior irradiation of normal tissues can significantly impair their ability to produce new vascular tissues in response to the tumor. Thus, drug delivery to the tumor can be reduced when tumors are grown in preirradiated sites. The "tumor bed effect" has been reviewed by Milas [54].

Techniques for the determination of *in vivo* antitumor activity

Several techniques have been used to determine the activity of antineoplastic agents against experimental tumors growing *in vivo*. These tend to fall into one of two main categories, *in situ* and excision assays. *In situ* assays are performed entirely *in vivo*. Tumors are inoculated into the appropriate host, treated *in vivo,* and the effects of drug treatment estimated either on various parameters of tumor growth, or on the duration of survival. Excision assays also are based on the treatment of tumors growing *in vivo*. However, the estimations of cytotoxicity require removal of the tumor for further evaluation. This can include estimating colony forming ability *in vitro*, or TD_{50} estimations of the number of treated cells required to form tumors on reinoculation into a second host. Each of these techniques has unique advantages and disadvantages. Irrespective of the tumor endpoint, many investigators include additional groups of tumor-bearing animals treated with a drug known to be active against the tumor model [67].

In situ assays — tumor growth delay

Perhaps the most widely used *in situ* technique determines the effect of drug treatment on the kinetics of tumor regrowth. Tumors are inoculated into the appropriate host, treatment being initiated when a specific tumor size is reached, and measured at regular intervals until both treated and untreated tumors have reached a predetermined size. Growth delay is assessed as the time difference between treated and control tumors to reach this predetermined size. This approach measures growth of tumors of the same size, an important consideration when Gompertzian kinetics are involved.

There are a number of considerations specific to the design of tumor growth delay assessments. The measurements should be performed on proliferating tumors, so that it is advisable to obtain some estimate of pretreatment tumor growth characteristics to enable tumor selection. For example, where the size at treatment is to be 6 mm, the range of tumor sizes for inclusion in combination chemotherapy studies might be ±1-2 mm in diameter. The issue of appropriate randomization of animals into each group is discussed elsewhere [68]. The optimal size at treatment will vary depending on the individual growth characteristics of the tumor cell line but should be sufficient to produce tumors that are easily measured yet not so large that they include significant areas of hypoxia or necrosis. The optimum endpoint size is close to the size at treatment, *e.g.* twice the treatment volume. This minimizes any effects of treatment on growth rate [69].

An alternative endpoint to growth delay for *in situ* techniques is overall survival. This method has proved reliable for the murine ascites tumors, since the growth properties and lethal tumor burden are well established. Survival can easily be compared with an untreated tumor-bearing population. Survival is a less reliable endpoint for many solid tumors. The majority of solid human breast tumor models exhibit a poor or unpredictable metastatic potential. Thus, tumor burden is almost exclusively provided by the primary tumor. In these cases, the lethal tumor burden (primary tumor), as a percent of total body weight, can be far in excess of that observed in humans. Some poorly vascularized tumors may increase in volume and yet contain a relatively stable volume of viable tissue.

Tumor measurement: area, volume, and weight

Tumor area and/or volume is usually recorded every 1-4 days, depending on the growth characteristics of the tumor. The length of the longest axis and the width perpendicular to the longest axis are sufficient to obtain tumor area. For easily accessible tumors, a third perpendicular measurement can be obtained to determine tumor volume. This is useful when it is apparent that palpable tumors are particularly irregular in shape and unlikely to meet the requirements for volume assessments from measurements of tumor area. For a more detailed description of tumor measurements see the accompanying article by Rygaard and Spang-Thomsen [70].

Tumor weight, as determined at necropsy, is used as an endpoint in some drug studies. However, it is generally inadvisable as the sole endpoint. While it provides an accurate measurement of the final tumor, it does not allow for assessments of the activity of the treatment on growth kinetics, being only a "snap shot" of the tumor's growth. For example, a cytotoxic and cytostatic treatment could produce the same reduction in final tumor weight. The cytotoxic treatment could have induced an initial complete remission (disappearance and regrowth of the tumor), implying a possible induction of cell death and the potential for an alternative dose/schedule to produce cure. The cytostatic agent could have decreased the rate of cell proliferation without inducing any significant cell kill. Where there is heterogeneity in initial cell volume, the ability to statistically demonstrate activity also may be compromised. This is less problematic when consecutive measurements are

obtained on each tumor, *e.g.* to obtain individual T_Ds.

It also is possible to estimate tumor weight/volume from caliper measurements of tumor area. Tumor volume can be obtained from area measurements by [71]:

$$\text{tumor weight (mg)} = (\text{length})\ (\text{width}^2) / 2$$

where: measurements = mm for all equations.

Where the volume is estimated at necropsy and the area of necrosis can be measured, this is modified to [72]:

$$\text{tumor weight (mg)} = [(\text{length} \times \text{width}^2) / 2] - [(\text{necrosis length} \times \text{necrosis width}^2) / 2]$$

For nonspherical tumors, the volume can be estimated by [73]:

$$\text{volume} = 4/3\pi(\text{largest diameter}/2 \times \text{smallest diameter}/2)^{3/2}$$

Other approaches include measuring three perpendicular dimensions and simply multiplying the three estimates [74]. While obtaining these measurements is often feasible with *s.c.* tumors in the flanks or back of nude mice, there can be difficulty in obtaining reproducible measurements of height for some tumors. The underlying assumption that the tumors are "box shaped" appears reasonable, since assuming an ellipsoid shape does not appear to give a better estimate, at least for human lung tumors [75].

Where necessary, the ellipsoid volume can be estimated by [76]:

$$\text{volume} = 4/3\pi(\text{length}/2 \times \text{width}/2 \times \text{height}/2)$$

When such tumor volume/weight estimates are used, it is advisable to confirm the validity of the relationship by at least comparing the predicted tumor weights/volumes with the actual wet weights/volumes at necropsy at the end of the experiment.

Tumor volume measurements can be used to estimate T_D and growth delay. Data also can be transformed and plotted as changes in relative tumor volume. Relative tumor volume can be obtained from [77]:

$$\text{relative volume} = V_a/V_i$$

where V_a = volume at the start of treatment, and V_i = volume at day i.

Excision assays

The main purpose of excision assays is to directly estimate the fraction of cells in a tumor that have retained their clonogenicity (fractional cell survival). The greatest methodological variation in these assays relates to the technique chosen to determine clonogenicity. Probably the more widely utilized clonogenicity assays determine the number of tumor cells, in an *ex vivo* suspension, able to form anchorage-independent colonies *in vitro*. Clonogenicity is usually assessed in a semi-solid medium (agar or methyl cellulose). This approach has significant advantages over other excision assays in time, cost, and intra-experimental variability [55], and in reducing the numbers of animals.

The TD_{50} assay determines tumorigenicity *in vivo* and, therefore, the tumorigenic potential of the treated cell populations. The excised tumor is disaggregated and a dilution cloning technique used to determine the minimum number of cells required to form tumors upon reinoculation. While immunologic properties of the tumor can be problematic, reinoculation into an immune-compromised host can largely eliminate these problems [55]. Ascites, such as the MDA435/LCC6 [21], can be inoculated *i.p.* and treated *i.v.*, *s.c.*, or *p.o.* Surviving cells can easily be removed and reinoculated either into the mammary fat pads of recipient mice to assess tumorigenicity as solid tumors, or *i.p.* to assess tumorigenicity by ascites formation. This approach, while providing a rigorous determination of effects on *in vivo* clonogenicity, can require relatively large numbers of animals depending on the study design.

The need to appropriately reduce animal usage is likely to effectively eliminate many of these *in vivo/in vivo* experimental approaches. However, a modification of this approach is to

assess clonogenicity of *in vivo* treated tumors *in vitro*. Thus, cells are treated as solid tumors or ascites *in vivo*, with cells removed and fractional cell survival determined by an anchorage-independent colony formation assay *in vitro* (above). This has the disadvantage that *in vitro* clonogenicity is used as a surrogate for tumorigenicity. Some cells also may not immediately readapt to *in vitro* growth in a manner that allows for an adequate assessment of cell survival. However, for many cellular models there is no significant effect on estimates of cell survival, but there are advantages in substantially reduced costs and fewer animals required.

All excision assays suffer from the disadvantage that, at some point, cells are removed from the animal for manipulation. Thus, the critical tumor/host relationship is lost. The disaggregation of solid tumors, for either *in vivo* or *in vitro* analysis, can result in a cell population no longer representative of the tumor. The process of enzymatic digestion can reduce clonogenic potential and thereby appear to increase the activity of the treatment. The time of excision can also influence the results, since some cells may eventually repair potentially lethal damage [78]. One advantage of an ascites model for these types of studies is that the cells may not require damaging enzymatic and/or physical disaggregation.

Endpoint comparisons

The most important technical disadvantage of the excision compared with the *in situ* techniques is the necessity for removal and manipulation of the tumor prior to assessing cytotoxicity. The loss of the tumor microenvironment also is problematic, since the ability of normal cells to stimulate tumor regrowth may be a crucial factor in the apparent failure of some cytotoxic regimens. However, excision assays eliminate the problem of remaining dead (or reproductively dead) cells contributing to tumor volume, a concern that can arise with *in situ* assays. The tumor growth delay assays have the disadvantage that the kinetics of tumor regrowth may be unpredictable for novel drug combinations. Tumors that regrow may have altered proportions of infiltrating normal cells that could cause an overestimation or underestimation of cell kill. The variability of intertumor regrowth patterns also can be problematic. Nevertheless, the *in situ* assays generally appear to be favored in the literature.

Death/survival as an endpoint

Many institutional animal care and use committees are restricting or eliminating death as an endpoint in drug screening and other *in vivo* studies. Nevertheless, some investigators may have experimental designs where death/morbidity/survival is a justified requirement of the study. Several related issues require consideration. Survival can be measured at a fixed time, *e.g.*, the proportion of animals remaining alive at a predetermined time point beyond when the last untreated animal dies, or the median duration of survival when all animals in all groups die within the observation time. When "cure" or the proportion of "long term survivors" are estimated, it is necessary to define the time point at which "cure" or "survival" is attributed. A major concern is the T_D, since sufficient time must be allowed for any significant number of remaining cells to proliferate to the point where a palpable tumor/ascites would be expected. A solid tumor with $T_D = 48$ hr may require up to four months of post treatment observation to establish "cure" [32]. Defining long term survivors (where death/morbidity is the primary endpoint) often is based on the duration of survival of mice bearing untreated tumors, *e.g.*, three times their mean or median survival. By this criterion, with a mean survival of 30 days in the untreated group, treated mice that survive for 90 days could be considered long term survivors [21]. When there are survivors, the data analysis procedures need to take this into account [68].

There is little ethical justification for using

death as an endpoint for solid tumors that are easily accessible for estimations of tumor growth delay or excision assays. In general, survival as an endpoint should probably be restricted to ascites models, and perhaps also to solid tumors known to achieve a lethal tumor burden within an appropriate and predictable time. Even for these tumors, it may be possible to substitute morbidity for death. We have found this to be a viable and more humane alternative in several ascites studies. While it requires knowledge of the time from the onset of morbidity to death, and evidence that this period is sufficiently consistent, this information can be obtained, in advance, on a relatively few animals.

Where possible, morbidity should be considered as a poetntial surrogate for death as an endpoint in survival analyses. To assist other investigators, the criteria used to define morbidity, and the verification of its applicability as a surrogate for death, should be reported.

Approaches to data analysis

Estimation of tumor doubling times

Estimates of T_D can be obtained from measurements of either tumor area or tumor volume. A detailed description of this approach can be found in the accompanying article by Rygaard & Spang-Thomsen [70]. The T_D for each individual tumor can be obtained, with those within an experimental group combined for further analysis, *e.g.*, use of an appropriate ANOVA to explore differences among different treatment groups. While there is evidence of investigators using simple linear regression models to estimate T_D from growth curves, this is potentially confounded by the Gompertzian nature of tumor growth kinetics [11,12,70]. However, in short term studies, particularly where the growth data approximate exponential growth, the Gompertzian model may reduce to an exponential growth model, which may provide a more efficient model for the data [68]. There also can be statistical concerns where early deaths limit the data available for the adequate determination of a T_D [68].

Cell kill estimates from in situ *studies*

There are a number of methods for estimating cell kill from *in situ* analyses. For ascites models, estimations of percent increased life span (%ILS) provide an indication of therapeutic activity. %ILS can be estimated from:

$$\%ILS = 100(T-C)/C$$

where: T = median survival time of treated populations; C = median survival time of untreated control population of tumor bearing mice [67].

It should be noted that median survival times focus on the 50% survival estimates, and do not efficiently use all of the data in the survival curve. This can become problematic when there is substantial heterogeneity in the duration of survival in some treatment groups. One approach is to use the hazard ratio (HR) of death for the control group *versus* the treated group (J Hanfelt, personal communication). Thus, where there is considerable heterogeneity in survival data, %ILS may be better estimated from:

$$\%ILS = 100(HR-1)$$

where: HR = the hazard ratio of death for the control group *versus* the treated group. The HR can be obtained by Cox proportional-hazards regression analysis [79,80].

Estimates of cell kill can be derived from solid tumor growth curves, where repeated measurements are obtained over the period of time required for tumors to reach a predetermined size. Provided the T_D for untreated and regrowing (treated) tumors are equivalent, the cell kill can be estimated by a number of related formulae [81]. Cell kill can be determined from growth delay measurements using an estimate of the T_D [9] and the number of cell divisions required for each log increase in growth (3.32) [82].

Total cell kill can be estimated from [81]:

$$\log_{10} \text{cell kill} = (T-C)/(3.32)(T_D)$$

Cell kill/dose can be estimated from [81]:

$$\log_{10} \text{ cell kill/dose} = (T\text{-}C)/(3.32)(T_D)(n)$$

where: T = median time to predetermined size in treated populations; C = median time to predetermined size in untreated control populations; n = number of treatments.

Net cell kill can be estimated from [81]:

$$\text{Net } \log_{10} \text{ cell kill} = (T\text{-}C)\text{–}(\text{duration of treatment})/(3.32)(T_D)$$

The specific growth delay for solid tumors can be estimated from the times taken for both treated (T) and control (C) tumors to reach a predetermined size [66]. If the specific growth delay value exceeds 2, the tumor model is often considered responsive. This is generally considered the most important estimate of antitumor activity, and can be reported along with the total cell kill estimated from the same primary data (above).

Specific growth delay can be estimated from [77,83]:

$$\text{Specific growth delay} = (T\text{-}C)/C$$

Where the T_D of tumor regrowth is significantly different from the T_D of untreated tumors, these estimates are invalid. For example, the T_D of treated populations may be slower than equivalent untreated tumors following irradiation [69]. Other limitations of these analyses are indicated when treatment is begun shortly after tumor cell inoculation, where a significant immune component is suspected, or where the treatments are close to being curative [72]. The degree of response in tumors that exhibit alterations in the T_D following treatment can be more appropriately expressed as delay/doubling time [69].

Determination of synergy

Studies are often performed to determine the nature of the interaction between two or more treatments. Many investigators claim synergistic interactions without having performed the necessary analyses. Several authors have more clearly defined these terms and described the conditions required to determine whether an interaction is synergistic, additive, or antagonistic [84-86].

Synergism is a mathematically defined interaction, most widely determined from isobologram analyses. Construction of classical isobolograms (isoeffect curves) for the determination of additivity, synergy, and antagonism generally requires an initial estimation of the dose response curve of each agent alone and dose responses of one agent in the presence of each of several concentrations of the second agent [72]. While this may be feasible for routine *in vitro* studies [84], classical isobologram analyses generally require too much information to be readily applicable to *in vivo* data. A minimum requirement for *in vivo* studies has been suggested, using five doses of each drug alone and three or four combination treatments using intermediate doses [87]. An interaction index (I_x) can then be estimated from the isobologram equation [88,89]:

$$I_x = (d/D) + (t/T)$$

where ED_n = some fixed level of activity, D and T = dose of each drug alone required to produce ED_n, and d and t = dose for each drug in combination that produce ED_n. I_x values of <1 indicate synergy, a value of 1 reflects additivity, while values >1 indicate antagonism. Ideally, each ED_n value should be estimated by performing appropriate probit or logit analyses of the dose reponse data [90,91].

An alternative experimental design is to fix the dose of the first drug and vary the concentration of the second [92]. This approach has not been widely applied to *in vivo* studies, and its ability to adequately define the nature of drug interactions does not appear to have been extensively confirmed. Nevertheless, it could be applied to *in vivo* studies and may be worthy of consideration.

Application of the median effect analysis is one of several alternative approaches to the classical isobologram approach for assessing synergy [85]. While this method may be amenable for use in *in vivo* studies, this has yet to be firmly

established. Other related approaches to assessing synergy also are reported [93] but their application to *in vivo* studies also remains to be confirmed. The concern is not the validity of the approaches, which is beyond the scope of the current article, but how appropriate *in vivo* experiments could be designed, within the constraints of time, cost, and appropriate animal usage, to produce sufficient data to generate statistically reliable estimates of the nature of the drug interactions.

Where one drug has no activity, synergy is more readily determined [93] and may simply require statistical evidence that the combination is significantly different from the inhibitory drug alone. This simplistic approach is potentially confounded when the "inactive" compound is active at higher concentrations. If the dose used was at a theoretical ED_{10}, *i.e.,* activity was present but could not be detected because of the sensitivity of the assay, assumptions of synergy could well be invalid. Thus, if this approach is to be used, it should be at least restricted to doses well below the minimally active dose, or better yet, restricted to studies where one compound is known to have no antineoplastic activity.

Where resources are available, isobologram-based approaches are preferred. However, some interactive definitions may be approximated from limited data as follows [94,95]:

Antagonistic	$(AB)/C > (A/C) \times (B/C)$
Additive	$(AB)/C = (A/C) \times (B/C)$
Synergistic	$(AB)/C < (A/C) \times (B/C)$

where A = response to treatment 1; B = response to treatment 2; C = response to no treatment/vehicle; AB = combination of treatments A and B. Some investigators define a fourth interaction category (subadditive = $(AB)/C > [(A/C) \times (B/C)] < [B/C]$ (where $B/C > A/C$). However, a subadditive interaction is essentially antagonistic, making the utility of this term somewhat unclear from a pharmacologic perspective.

The application of this approach may vary depending on the endpoint and experimental design chosen for a particular study. For excision assays using *in vitro* colony formation as an endpoint, the response to treatment would be represented by the colony forming ability or surviving fraction. Growth delay assays could utilize the estimates of cell kill/survival described above or T_D parameters. A description of approaches to the statistical analysis of data obtained from these studies can be found in the accompanying article by Hanfelt [68].

These terms are approximations, at best, when compared with more classical isobologram approaches, and should be used with some caution. The outcome provides only a general approximation of the nature of the interaction, and should not be considered definitive. While reasonable approximations may be obtained where tumors approximate logarithmic growth, the analysis does not take into account the shape of the respective dose response curves, a central component of isobologram approaches for determining the nature of drug interactions. This could lead to both underestimates and overestimates of potential synergy or antagonism.

Therapeutic synergism has been defined as occurring when the response of a combination is in excess of the maximum response of either drug alone (*e.g.* A = 100%, B = 150%, A+B = 200%) at equitoxic doses [67,96,97]. The term "clinical synergism" also has been applied when a combination chemotherapy regimen is curative [96]. These are not particularly informative definitions from an interpretive or mechanistic perspective, and could be applicable when the "true" biochemical interaction is only additive or even antagonistic.

In practice, a drug combination can be curative and yet produce a nonsynergistic interaction. If two drugs with different mechanisms of action each kill 10^6 cells, a tumor of 10^9 cells could still be cured by an additive or even antagonistic interaction [96]. Thus, the determination of synergy or antagonism is not required where the purpose is simply to show that a combination of two or more drugs is curative relative to either agent administered alone.

The toxicity of a particular combination should be considered when determining the nature of an interaction between two or more drugs. In order to make appropriate comparisons, the treatments should be approximately equitoxic [97]. For example, the improved antitumor activity of a combination regimen which is accompanied by significantly increased toxicity might be no more effective than a higher dose of either agent alone that produced equivalent toxicity.

Some suggestions for statistical approaches to data analysis and the assessment of activity

The statistical analytical procedures used to explore data from animal studies is dependent on the study design. Statistical approaches will be dealt with only cursorily, since a detailed discussion of methods and approaches is provided elsewhere in this issue [68]. Readers are encouraged to consult this and other articles which deal with endpoints and considerations in depth beyond the scope of this section. While the use of some specific tests is suggested below, these should be used with some caution, since the primary data may violate assumptions implicit within the analyses. For example, some of these tests described below require that the data approximate a normal or other distributional form. When this is not apparent, or cannot be achieved by transformation, *e.g.* converting to $\log_{10}$, nonparametric analyses may be required. For investigators unfamiliar with statistical analyses, there is no substitution for consultation with a biostatistician.

One of the key first steps in the design of animal experiments should be the determination of appropriate group sizes. The number of animals per group will depend on the endpoint and the magnitude of the expected response. In this regard, it is necessary to perform appropriate power estimates to ensure that the design will have sufficient statistical power to adequately identify significant or nonsignificant differences. This is discussed in some detail in the accompanying article by Hanfelt [68]. It is important to ensure that group size is sufficient to maintain power when some animals may either die from other causes, *e.g.* drug toxicity or secondary infection, or bear tumors that do not exhibit appropriate pretreatment kinetics. For pilot/exploratory studies, where more definitive follow-up studies are planned, this is of lesser concern. The data from such pilot studies can often provide useful information on the expected response rate and toxicity, thereby facilitating the generation of potentially more relevant power estimates.

Tumor growth delay is generally assessed in terms of the time necessary for tumor growth in each group to increase from the initial size at the time of treatment to a larger, predetermined size [66,81,98]. The most common approaches are to compare the T/C or T-C values. Both parameters are usually based on median values, with zeros included for the T/C estimates and "tumor-free cures/tumor free survivors" being excluded from the estimates and analyzed separately. A T/C value $\leq 42\%$ is generally required to demonstrate activity [67]. The necessity to exclude cures may be problematic if it affects the power of the analysis. As discussed by Hanfelt [68], the use of median times also may be inefficient, with longitudinal analyses providing a more effective use of the data.

There are several other ways to explore data from tumor growth delay studies. For example, survival analyses also can be used to assess "time-to-event" endpoints, *e.g.* time to reach a predetermined size. A further approach is to use a repeated measures ANOVA to compare tumor size at each time point across the analysis. This may be most applicable if a significant number of tumors in a treatment group do not reach the predetermined size in the control group. Alternatively, tumor doubling times can be estimated for each individual tumor in each group by applying Gompertzian kinetic analyses, such as those performed by the GROWTH software [70]. Tumor doubling times can be compared among experimental groups by either ANOVA or multivariate ANOVA [99]. This approach also is useful for

cytostatic treatments, *e.g.,* hormones or anti-hormones, and to ensure that the kinetics of regrowth are appropriate for cell kill estimates.

For tumor excision assays, *in vitro* colony forming ability among groups, or *in vivo* tumor incidence if reinoculated into recipient mice, can be compared by either ANOVA or multivariate ANOVA.

Percent increased life span (%ILS) is the most widely used activity measure for ascites tumors. Since some animals may survive, survival curves may be estimated using the Kaplan-Meier approach [100], and differences in survival between treatment groups estimated by the Log-Rank test [101]. Where early deaths occur from drug toxicity and later deaths from tumor burden, a model more sensitive to early events may be necessary [68]. In general, a %ILS ≥27% in the P388 ascites model is considered the minimum for activity when both drug and tumor are administered *i.p.* When drugs are administered *i.v.* to an *i.p.* tumor, a %ILS >40% is considered sufficient to demonstrate activity [67].

Tumor incidence, proportion of survivors, or long term survivors, *e.g.* 2x2 analysis using single treatment and control, can be compared among groups by χ^2. If more than one site is used per mouse, General Estimating Equation methods are required to account for any lack of independence of tumors within animals [68]. If the number of observations is small, a Fishers exact test or Pearson's χ^2 test may be used.

Body or tumor weights, where the data are continuous and randomly distributed, can be compared by ANOVA followed by a multiple comparison *post hoc* test such as Duncan's multiple range test [102]. Where it is required to merely compare several individual groups with the same control, *e.g.* dose-response analysis, and where the group sizes are equal, Dunnet's t-test can be used [103]. Where group sizes are different, Scheffe's multiple comparison test can be applied [104]. However, it should be noted that some endpoints also are associated with several variables. For example, organ weights increase with increasing body weight. For such endpoints, analysis of covariance approaches are required [105].

Conclusions

As indicated in the introduction, many of the issues are raised to assist investigators in approaching some of the major concerns that arise in designing studies to test the activity of cytotoxic agents. There are alternative approaches to several of these issues, but a detailed discussion is beyond the scope of the current article. Some are described in detail elsewhere in this journal issue, and others can be found in many of the publications cited herein. While the relative importance of many of the topics addressed will vary with the hypothesis and endpoint chosen, some issues will apply irrespective of these, *e.g.* the need to design experiments with sufficient statistical power.

Acknowledgments

The author wishes to thank Drs. John Hanfelt, Fabio Lenoness, and Robert Glazer for critical reading of the manuscript. This work was supported by research grant DAMD17-96-1-6231 from the U.S. Department of Defense, U.S. Army Medical Research and Materiel Command.

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